REMARKS

Receipt of the Office Action, mailed August 25, 2003, is acknowledged. Applicants respectfully request reconsideration of the present application in view of the reasons that follow.

Claims 18-24 and 26-33 are pending in this application. Of these, claims 26 and 27 are withdrawn from consideration and claims 18-24 and 28-33 stand rejected. Under 35 U.S.C. § 112, ¶ 1, the Examiner rejected claims 18-24 and 28-33 for alleged "non-enablement," and claims 30-33 separately, for an asserted lack of "written description." Applicants respectfully traverse these rejections, while noting, with appreciation, the withdrawal of all other rejections.

I. The Specification Enables the Methods Claimed

As to claims 18-24 and 28-33, the Examiner asserts that "modifying learning and facilitating memory retrieval" or treating "dementia," as recited, is not enabled because the "quantity of experimentation required to use *LVV-Hemorphin-7* to treat a neuronal condition in humans or animals is immense." In addition, the Examiner contends that the skilled person would not know what "neurological condition ... scopolamine is intended to model." Specifically, the Examiner finds it "difficult to imagine many human syndromes that would correlate to the rat model of temporary amnesia presented."

The first paragraph of Section 112 requires applicants to provide a specification that enables a person reasonably skilled in the art to make and use the claimed invention without undue experimentation. The fact that some experimentation may be employed, however, does not make it undue if a person of skill in the art typically engages in such experimentation. This is because the prohibition is against "undue experimentation," not merely "experimentation." *In re Angstadt,* 537 F.2d 498, 502-3 (CCPA 1976).

The Examiner acknowledges that the specification shows that the "administration LVV-Hemorphin-7 reverse[s] the temporary amnesia caused by scopolamine" and that the drug would likely "improve the performance on tasks used to study short-term memory, acute memory, or consolidation to long-term memory, when such memory processes are inhibited by scopolamine." Applicants agree with the Examiner's statements and reaffirm that this disclosure was made by pointing to the specification, which includes specific examples of tests conducted on rats in passive avoidance tests and in the circular water maze test, indicating that LVV-Hemorphin-7 reversed the temporary memory loss caused by scopolamine. See Examples 11 and 12.

The Examiner, however, contends that the amount of experimentation necessary to use *LVV-Hemorphin-7* to treat neuronal conditions in humans or other animals is undue because persons of skill in the art would not have "known what condition scopolamine is intended to model." This statement is not factually supported. At the time the application was filed, those skilled in the art would have known that the scopolamine model is an accepted model for amnesia, dementia, and other memory-related losses in humans and other animals. Thus, the specification enables the modification of a whole range of biological activities in mammals, as presently recited, and not just reversal of temporary, scopolamine-induced memory loss, as the Examiner posits.

To evidence what the skilled person knows in the relevant art, applicants provide the declaration of co-inventor Siew Yeen Chai. In paragraph 13 of her declaration, for instance, Dr. Chai points to several instances in the literature that indicate the usefulness of scopolamine as a model for memory-related losses, including amnesia and dementia, in humans as well as other animals. Dr. Chai also identifies studies that illustrate how scopolamine impairs learning and memory under a variety of testing conditions, and how some of these impairments share neuropsychological similarities with impairments attributed to Alzheimer's disease.

Armed with the tests and methods disclosed in the present specification, therefore, one of skill in the art would need only routine experimentation, employing the accepted animal model, in order to use *LVV-Hemorphin-7* to modify "learning," facilitate "memory retrieval," and treat "dementia" in mammals as claimed. Moreover, no "undue" experimentation would pertain to the skilled person's modulating a mammal's biological activity, as claimed; this, in view of fact that the scopolamine model, employed by applicants and described in the specification, is a well-known representation of mammalian memory losses. Conversely, the Examiner has failed to identify what information is missing from the specification and why one skilled in the art otherwise could not make and use the claimed invention without undue experimentation. The Examiner has failed to make out a *prima facie* case of non-enablement, therefore, which warrants withdrawal of this rejection.

II. The Written Description Supports the Claims

The Examiner also rejected claims 30-33 because the "specification as originally filed does not provide adequate written description for an isolated peptide of 10 amino acids wherein one or more of the amino acids are modified, while still maintaining the function of the *LVV-Hemorphin-7* polypeptide." In other words, the Examiner contends that these "modifications" are too speculative.

The Examiner's quoted rationale does not relate to the claims, however. The Examiner improperly requires the applicants to describe "an isolated peptide of 10 amino acids wherein one or more of the amino acids are modified." *See* Office Action. The claims, however, do not recite this limitation. Claim 32, for example, recites a method of administering the neuroactive peptide of SEQ ID NO:1, wherein the neuroactive peptide has "one or more non-naturally occurring amino acids, and/or amino acid analogues." This method is what must be described, and what applicants submit is described, in the specification.

Even at face value, the Examiner's contentions also do not apply to claims 30 and 31, because the recited "D-amino acids" do not represent a "modification" from the native primary sequence, as the Examiner is heard to urge, but merely a change in chirality vis-à-vis residues of the native sequence. In any event, one of skill in the art would recognize that applicants invented *LVV-Hemorphin-7* peptides that contain one or more D-amino acids. These claims are not unduly speculative, in other words. The specification describes these peptides and describes the use of D-amino acids. *See* page 3, line 29-30. Moreover, the sequence of the peptide recited in the claim is the same as SEQ ID NO:1 disclosed in the specification. The only difference is that one or more of the amino acids in the sequence has a particular chirality making it a D-amino acid.

The chemistry associated with the D-amino acids and with the changes in chirality are well-known by those with skill in the art. In the attached declaration, Dr. Chai points to numerous articles and patents that describe the use of D-amino acids. Thus, one of skill in the art would understand the meaning of the phrase "D-amino acids" and would recognize that the applicants were in possession of *LVV-Hemorphin-7* peptides that contain one or more D-amino acids. Moreover, one of skill in the art would understand that D-amino acids can be produced by standard techniques well known in the art and can be tested in the functional assays described in the present patent application in Example 7. Thus, these peptides are fully described in the specification. Applicants respectfully request that the Examiner reconsider and withdraw this rejection with respect to claims 30 and 31.

With respect to claims 32 and 33, one of skill in the art would also recognize that applicants were in possession of the neuroactive peptide of SEQ ID NO: 1 that contains one or more non-naturally occurring amino acids, and/or amino acid analogues as claimed. The specification discloses the sequence of SEQ ID NO: 1. Those with skill in the art know the chemistry related to peptides and amino acids well and know that the methods for changing amino acids in a peptide to non-naturally occurring amino acids are routine in nature. *See* page 4, ll. 32-37. In addition, the specification

describes how *LVV-Hemorphin-7* effectuates its memory enhancing effect through its affinity to the AT_4 receptor and how modifying the sequence can improve the activity. *See* page 5, ll. 6-12. The original specification even says that "[u]sing modern methods of peptide synthesis and combinatorial chemistry, it is possible to synthesize and test very large numbers of analogues within a short period of time, and such synthesis and screening is routinely carried out by pharmaceutical companies" (page 4, ll. 32-37). In Example 7, the specification describes a functional assay for screening *LVV-Hemorphin-7* and analogues for binding to the AT_4 receptor. Thus, analogues, such as those recited in claims 32 and 33, can easily be produced and tested in order to determine whether the analogue would be able to "modulat[e] neuronal activity that mediates said biological activity" as claimed. Thus, those of skill in the art would understand, based on the specification, that the applicants invented the neuroactive peptides recited in claims 32 and 33.

In support this argument, Dr. Chai comments on Lee *et al.* (2003), 305(1):205-11 ("Lee") (copy enclosed), which describes structural activity studies conducted on the fragments of *LVV-Hemorphin-7* on the AT_4 receptor binding assay to determine important amino acid residues for binding and for memory-enhancing activity. Further, Dr. Chai describes how the functional assays have also been performed on *LVV-Hemorphin-7* analogues, as described in Lee, where peptides comprising consecutive alanine substitutions of the amino acid residues of *LVV-Hemorphin-7* were subjected to the AT_4 receptor binding assays. *See* attached declaration by Dr. Chai. Dr. Chai concludes that the data and tests disclosed in Lee are routine in nature and could have been completed by one of ordinary skill in the art. Therefore, the specification describes the subject matter claimed in claims 32 and 33 so as to allow persons of skill in the art to recognize that the applicants invented what is claimed. Applicants respectfully request that the Examiner reconsider and withdraw this rejection with respect to claims 32 and 33.

In light of the foregoing, applicants believe that the present application is in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested. The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicants hereby petition for such extension under 37 C.F.R. § 1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date 26 January 2004

By S. A. Bent

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Stephen A. Bent
Attorney for Applicant
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PATENT IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Mendelsohn, et al	Examiner:	Wegert, Sandra
Serial No:	09/147,490	Group Art Unit:	1647
Filed:	05/13/1999	Docket No.:	016786/0215
Title:	Neuroactive Peptide		

CERTIFICATE UNDER 37 CFR 1.8: The undersigned hereby certifies that this correspondence is being deposited in the United States Postal Service, as first class mail, in an envelope addressed to:
Assistant Commissioner for patents, Washington, D.C. 20231 on _____

By: _____
Name:

AFFIDAVIT OF DR SIEW YEEN CHAI

BOX AF

Assistant Commissioner for Patents
Washington D.C. 20231

Dear Sir:

I, Dr Siew Yeen Chai declare and state:

1. That my professional training and experience are documented on the abridged *curriculum vitae* attached as Exhibit 1.

2. That I am currently employed as a Senior Research Fellow, Howard Florey Research Institute of Experimental Physiology and Medicine ("my company"), which is located in Melbourne, Victoria, Australia. My past employment history is documented on the attached abridged *curriculum vitae.*

3. I am one of the applicants and inventor of the invention covered by United States Patent Application Serial No. 09/147,490.

4. I have read and considered the Examiners Office Action issued on August 12, 2003, the specification of 09/147,490 and the currently pending claims 18 to 24 and 26 to 33.

5. In particular I have read the Examiners comments regarding the enablement and written description of the claimed invention. I note that the Examiner contends on pages 3 to 5 of the Office Action that "it is difficult to imagine many human syndromes that would correlate with the rat model of temporary amnesia presented."

Further, at page 5, second para I note that the Examiner states that "undue experimentation would be required to determine a disease for which scopolamine administration was an accurate model."

6. Firstly, as appreciated by most scientists, animal models are only models and do not mimic completely particular human conditions. However, this does not mean that these are not useful or predictive of human conditions. For example, the review by Gallagher & Rapp (1997), entitled "The Use of Animal Models to Study the Effects of Aging on Cognition" (Annual Review of Psychology, Vol. 48, pp. 339-370) describes the importance of animal models for understanding the effects of normal aging on the brain and cognitive functions. (Copy enclosed).

7. Secondly, with respect to the scopolamine-induced amnesia model for dementia, I totally disagree with the Examiner's contention and suggest that there are significant amounts of evidence in the literature to support the usefulness of this model for dementia, as amnesia is the most predominant debilitating symptom of dementia. However, before discussing these further it is worthwhile reviewing the background of the present invention.

8. The characteristic features seen in the brain of Alzheimer's disease sufferers are amyloid plaques and neurofibrillary tangles. Amyloid plaques are insoluble protein deposits that accumulate in areas of the brain used for memory, ie. the hippocampus and related structures. The plaques are mainly composed of a partial beta-pleated sheet polypeptide, called beta-amyloid (ßA). Neurofibrillary tangles are insoluble paired helical filaments composed of the microtubule-associated protein tau.

9. Many researchers suggest that Alzheimer's disease triggers the overproduction of the enzyme acetylcholinesterase (cholinesterase), that catalyses the breakdown of acetylcholine. Acetylcholine is a neurotransmitter that plays an important role in processing learning and memory and therefore its breakdown disrupts nerve communication in the brain, which causes cognitive decline and other memory problems. See, for example, Blokland (1995), Brain Res Rev.;21(3):285-300, copy enclosed).

10. More importantly, it is now widely accepted that there is a link between acetylcholine and the deposition of amyloid plaques. For example, see Isacson et al. 2002, Trends in Neuroscience 25(2):79-84; copy enclosed).

11. Furthermore, the formation of neuritic plaques and neurofibrillary tangles and degeneration of cholinergic neurones are neuropathological characteristic of brains of individuals with Alzheimer's dementia Davies P, Maloney AJF (1976) Selective loss of central cholinergic neurons in Alzheimer's disease. Lancet i: 1403; Isacson O, Seo H, Lin L, Albeck D and Granholm AC (2002) Alzheimer's disease and Down's syndrome: roles of APP, trophic factors and ACh. Trends in Neuroscience 25(2):79-84.

12. It is noteworthy that all current FDA approved drugs for the treatment of dementia, including Alzheimer's, are acetylcholinesterase inhibitors ie they prevent the breakdown of acetylcholine. Therefore, it can be seen that any animal model, which mimics the disruption in acetylcholine production or function, potentially

mimics the formation of amyloid plaques and/or neurofibrillary tangles.

13. Scopolamine is an acetylcholine receptor antagonist, which blocks the function of acetylcholine. Scopolamine has been shown in numerous studies to impair learning and memory under a variety of testing conditions, not only in small animals (Spencer and Lal, 1973), but also in monkeys (Ogura and Aigner, 1993; Rupniak *et al.*, 1989) and in human (Ghoneim and Mewaldt, 1977; Rusted and Warburton, 1988). Some of these impairments reflect neuropsychological similarities with the demented states in patients with Alzheimer's disease (Molchan *et al.*, 1992). Copies of these documents are enclosed.

14. Numerous articles relating to the use of the scopolamine-induced amnesia model for dementia have been published. For example, Naveen & Kohli (2003), Indian Journal of Pharmacology, 35: 104-108; Ye *et al.* (1999), Journal of Pharmacol Exp Ther; 288(2):814-9; Ebert & Kirch (1998), European Journal of Clinical Investigation, Volume 28 Issue 11 page 944. Copies of these documents are enclosed.

15. Consequently, based on the above background material, I believe that the scopolamine-induced amnesia model for dementia is valid and useful. More importantly, I believe that the literature in this area supports my view that this model is predictive of human dementia and as such the Examiner's comments regarding this model should be put aside.

16. With respect to teaching of 09/147,490, I note that the Examiner refers to LVV-Hemorphin-7 as "a scopolamine inhibitor" on page 5, line 18. As stated throughout the specification (eg page 1, line 3; page 19, lines 3 to 5) LVV-Hemorphin-7 is a 10 amino acid peptide found in the brain, pituitary, hypothalamus and bone marrow that binds to the Angiotensin AT_4 receptor (see Examples 6 and 7). It ***does not*** act as an inhibitor of scopolamine. Angiotensin IV (VYIHPF) has been shown to reverse amnesia induced by other interventions, including (1) bilateral perforant pathway knife cuts (Wright *et al.* (2002), J. Neurosci 19(10):3952-3961) and (2) global ischemia Wright *et al.* (1996), Brain Res. 717:1-11. Accordingly, by binding to the AT_4 receptor LVV-Hemorphin-7 is able to mimic the actions of Angiotensin IV thereby mediating the effects on learning and memory and bring about amnesia reversal. Because of its actions my co-inventors and I have designated LVV-Hemorphin-7 as another AT_4 receptor ligand that is structurally distinct from Angiotensin IV.

17. In conclusion, I believe that the scopolamine-induced amnesia model for dementia is valid. The specification discloses that LVV-Hemorphin-7 is able to mimic the actions of Angiotensin IV and bring about amnesia reversal. Accordingly, the specification teaches that LVV-Hemorphin-7 can be administered to dementia patients to alleviate memory loss. Other aspects, such as routes of administration, formulation and the like are well within the scope of the skilled addressee.

18. With respect, to the rejection of claims 30-33 based on the amino acid substitution of LVV-hemorphin 7 having a memory-enhancing effect, I provide a copy of Lee *et al.* (2003). As discussed above, LVV-hemorphin 7 mediates its memory enhancing effect by its high affinity binding to the AT_4 receptor. Example 7, and elsewhere in the patent specification, describes a functional assay for screening

LVV-hemorphin 7 and analogues' for binding to the AT_4 receptor. Functional assays have now been performed on LVV-hemorphin 7 analogues – Our publication Lee *et al.* (2003) "Structure-activity study of LVV-Hemorphin-7:angiotensin AT_4 receptor ligand and inhibitor of insulin-regulated aminopeptidase", J Pharmacol Exp Therap 305(1):205-211, where peptides comprising of consecutive alanine substitutions of the amino acid residues of LVV-hemorphin 7 were subjected to the AT_4 receptor binding assays.

19. I believe that those data disclosed in Lee *et al.* (2003) were routine in nature and could have been produced by anyone skilled in the art. Indeed, techniques like alanine scanning have been routinely applied in most molecular biology laboratories since the late-1980's to determine important amino acids. Having identified important amino acids, analogues could be produced and tested in order to assess their binding activity.

20. With respect to the use of D-amino acids these are well within the skill of any person in this field. For example, US patents 6,656,700, 6,635,739 and 6,458,357 describes the use of D-amino acids, which are more resistant to proteolytic attack. Use of such D amino acids is also referred to in Kole *et al.*, Biochem. Biophys. Res. Com. 209:817-821 (1995).

21. In conclusion, I consider that amino acid variants of the LVV-hemorphin 7 molecule, including D amino acid variants, could be readily produced by standard techniques well known in the art. These could be also be readily tested in the functional assays described in 09/147,490.

22. I declare that all statements made herein on my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful, false statements may jeopardise the validity of the application or patent issuing therefrom.

Respectfully submitted,

By: _____ Date: 13TH JAN 2004

Name: Doctor Siew Yeen Chai

Title: Senior Research Fellow

IN THE MATTER OF
US Serial No: 09/147,490
entitled"Neuroactive Peptide"

EXHIBIT 1

This is Exhibit 1 referred to in Clause 1 of the Statutory Declaration Siew Yeen Chai dated 13th Day of January 2004.

Before me: _____

DR S.J. BOYER
GRIFFITH HACK
Level 6/256 Adelaide Tce, Perth 6000
A Registered Patent Attorney within the
meaning of the Patents Act 1990.

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meaning of the Patents Act 1990.

CURRICULUM VITAE

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EDUCATION: **Tertiary:**
(1) Bachelor of Science (Honours), 1980-1983
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(2) Doctor of Philosophy, 1984-1988
University of Melbourne, Parkville, Victoria, Australia

AWARDS:
(1) University of Melbourne, Special Postgraduate Studentship
(2) National Health and Medical Research Council (NHMRC),
CJ Martin Postdoctoral Fellowship
(3) National Health and Medical Research Council (NHMRC),
Australian Postdoctoral Fellowship

CURRENT APPOINTMENTS:

NHMRC Senior Research Fellow, Howard Florey Institute
Honorary Research Fellow, Department of Anatomy and Cell Biology, The University of Melbourne

Group Leader : Molecular Neurochemistry group

My research group comprises of

- Dr Anthony L. Albiston — Senior Research Officer 6
- Dr Trisha A. Jenkins — NHMRC Howard Florey Centenary Fellow
- Ms Ruani Fernando — PhD student
- Ms Siying Ye — PhD student
- Mr Grantley Peck — PhD student

PREVIOUS APPOINTMENTS:

1983
Student - Bachelor of Science, Honours
Department of Pharmacology, Monash University
Supervisors: Drs. Lesley Rogers & Roger King
Honours thesis entitled "The effects of pretreatment of diazepam on two behavioural changes induced by glutamate".

1984-1988
Student - Doctor of Philosophy,
University of Melbourne, Department of Medicine, Austin Hospital
Supervisor: Professor Frederick A.O. Mendelsohn
PhD thesis entitled "Localization of angiotensin converting enzyme in brain and peripheral tissues".

1987
Visiting Scientist,
Department of Histology, Karolinska Institute, Stockholm, Sweden
with Professor Tomas Hokfelt

1988-1990
Research Officer,
University of Melbourne, Department of Medicine, Austin Hospital
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1991-1992
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1992-1995
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1996
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1997-1998
Senior Research Officer
Howard Florey Institute of Experimental Physiology & Medicine

1999-2003
NHMRC Research Fellow
Howard Florey Institute of Experimental Physiology & Medicine

LEAVE OF ABSENCE:

(1) Maternity leave April 1990-June 1991
(2) Maternity leave January 1993-October 1993

PROFESSIONAL ACTIVITIES:

(A) MEMBERSHIP OF SOCIETIES

(1) Australian Society for Medical Research
(2) Australian Neuroscience Society
(3) Society for Neuroscience, USA
(4) International Society for Neurochemistry
(5) National Association of Research Fellows

(B) GRADUATE STUDENTS SUPERVISED:

(1) Ingrid Moeller PhD (1993-1996) University of Melbourne
Localization and function of the AT_4 receptor (passed 1996)
Recipient of the NHMRC Dora Lush Biomedical Scholarship
Currently a senior research officer at the School of Pharmacy, University of Queensland

(2) Trish Jenkins PhD (1994-1997) University of Melbourne
Interactions between angiotensin and brain dopamine (passed 1997)
Recipient of the NHMRC Dora Lush Biomedical Scholarship
Currently a postdoctoral research fellow at the Department of Psychology, University of Cardiff

(3) Carmel Murone PhD (1995-1998) University of Melbourne
Bradykinin receptors - localization, characterization and regulation (passed 1999)
Recipient of the Lesley Eric Paddle Scholarship
Currently a research scientist at the Ludwig Institute for Cancer Research, Austin and Repatriation Medical Centre

(4) Linda Wildemors B. Pharm (1996-1997) University of Groningen
Interaction of angiotensin II with dopamine in the shell of the nucleus accumbens

(5) Joo Hyung Lee PhD (1998-2002) University of Melbourne
Interaction of angiotensin IV and LVV-hemorphin 7 with acetylcholine (passed 2002)
Recipient of the University of Melbourne, Medical Research Scholarship
Currently a postdoctoral research fellow in the Division of Applied and Interventional Research at Toronto Western Hospital

(6) Ruani Fernando PhD (2002-) University of Melbourne
Cellular characterisation of insulin-regulated aminopeptidase (IRAP)
Recipient of the Australian Postgraduate Scholarship

(7) Grantley Peck PhD (2002-) University of Melbourne
Role of insulin-regulated aminopeptidase (IRAP) in the brain
Recipient of the Howard Florey Institute PhD Scholarship

(8) Siying Ye PhD (2002-) University of Melbourne
Functional studies on insulin-regulated aminopeptidase (IRAP)

© INTERNATIONAL INVITED PRESENTATIONS:

- Gordon Research Conference On Angiotensin
  Harbourtown Resort, Ventura, California, USA, February 1998
  Symposium entitled "The Angiotensin AT_4 receptor" (Organizer Prof J. Harding)
  Presentation entitled "Isolation of a neuropeptide with high affinity for the AT_4 receptor: LVV-hemorphin 7".

- Roche Biosciences
  Palo Alto, California, USA, May 2002 (Host Dr K. Chang)
  Redefining the angiotensin AT_4 receptors: what it is and what it does.

- Institut de Recherches Servier,
  Croissy sur Seine, Paris, France, May 2002 (Host Dr P. Renard)
  Redefining the angiotensin AT_4 receptors: what it is and what it does.

- Schering AG
  Berlin, Germany, May 2002 (Host Dr B. Seilheimer)
  Redefining the angiotensin AT_4 receptors: what it is and what it does.

- Department of Physiology, Faculty of Medicine, Chinese University of Hong Kong, December 2002 (Host Dr P.S. Leung)
  Redefining the angiotensin AT_4 receptors: what it is and what it does.

- 3rd General Meeting of the International Proteolysis Society
  Nagoya, Japan, November 2003
  Symposium entitled "Regulation of IRAP/P-LAP/A-LAP" (Organizer Dr S. Keller)
  Presentation entitled "IRAP is the AT_4 receptor".

- Gordon Research Conference On Angiotensin

Harbourtown Resort, Ventura, California, USA, February 2004
Symposium entitled "The Angiotensin AT_4 receptor" (Organizer Prof F.A.O. Mendelsohn)
Presentation entitled "The Angiotensin AT_4 receptor is Insulin-regulated Aminopeptidase IRAP".

PATENTS HELD:

1. Mendelsohn, F.A.O., Chai, S.Y., Aldred, G.P., Moeller, I., Smith, I., and Lew, R. "Neuroactive peptide"
   - Australian Patent No. 737386 (granted - commencing 9th July 1997)
   - NZ Patent Application No. 333632 (granted)
   - US Patent Application No. 09/147490 (appeal pending)
   - Japanese Patent Application No. 504586/1998 (under examination)

2. Albiston, A.L., McDowall, S.G., Mendelsohn, F.A.O. and Chai, S.Y. "Modulation of aminopeptidase activity"
   - Australian Provisional Patent PR6772 (filed 2nd August 2001)
   - US Provisional Patent Application No. 60/330170 (filed 17th October 2001)
   - PCT filed 2nd August 2002

PUBLICATIONS

(A) Refereed Journals

1. Mendelsohn, F. A. O., **S. Y. Chai,** and M. Dunbar. 1984. In vitro autoradiographic localization of angiotensin converting enzyme in rat brain using 125I-labelled MK351A. *Journal of Hypertension* 2(suppl 3):41-44.

2. Allen, R. K. A., **S. Y. Chai,** M. Dunbar, and F. A. O. Mendelsohn. 1986. In vitro autoradiographic localization of angiotensin converting enzyme in sarcoid lymph nodes. *Chest* 90:315-320.

3. **Chai, S. Y.,** P. M. Sexton, A. M. Allen, R. Figdor, and F. A. O. Mendelsohn. 1986. In vitro autoradiographic localization of ANP receptors in rat kidney and adrenal gland. *American Journal of Physiology* 250:F753-F757.

4. **Chai, S. Y.,** G. Paxinos, and F. A. O. Mendelsohn. 1987. Angiotensin converting enzyme in rat brain visualized by in vitro autoradiography. *Neuroscience* 20(2):615-627.

5. **Chai, S. Y.,** M. J. Christie, P. M. Beart, and F. A. O. Mendelsohn. 1987. Effects of nigral dopaminergic lesions and striatal excitotoxin lesions on brain converting enzyme. *Neurochemistry International* 10(1):101-107.

6. **Chai, S. Y.,** M. J. McKinley, and F. A. O. Mendelsohn. 1987. Distribution of angiotensin converting enzyme in sheep hypothalamus and medulla oblongata

visualized by in vitro autoradiography. *Clinical and Experimental Hypertension* A9(2&3):449-460.

7. Sakaguchi, K., **S. Y. Chai,** B. Jackson, C. I. Johnston, and F. A. O. Mendelsohn. 1987. Blockade of angiotensin converting enzyme in circumventricular organs of the brain after oral lisinopril administration demonstrated by quantitative in vitro autoradiography. *Clinical and Experimental Pharmacology and Physiology* 14:155-158.

8. Allen, A. M., **S. Y. Chai,** J. Clevers, M. J. McKinley, G. Paxinos, and F. A. O. Mendelsohn. 1988. Localization and characterization of angiotensin II binding and angiotensin converting enzyme in the human medulla oblongata. *Journal of Comparative Neurology* 269:249-264.

9. Sakaguchi, K., **S. Y. Chai,** B. Jackson, C. I. Johnston, and F. A. O. Mendelsohn. 1988. Inhibition of tissue angiotensin converting enzyme : quantitation by autoradiography. *Hypertension* 11(3):230-238.

10. Sakaguchi, K., B. Jackson, **S. Y. Chai,** F. A. O. Mendelsohn, and C. I. Johnston. 1988. Effects of perindopril on tissue angiotensin converting enzyme activity demonstrated by quantitative in vitro autoradiography. *Journal of Cardiovascular Pharmacology* 12:710-718.

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(C) Submitted Manuscripts

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(D) Submitted Reviews

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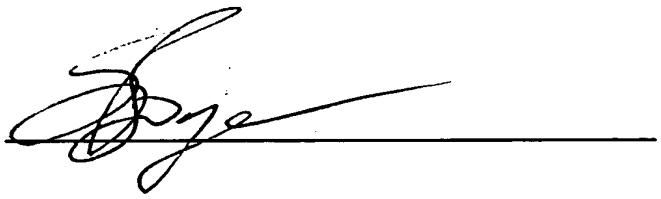
IN THE MATTER OF
US Serial No: 09/147,490
entitled"Neuroactive Peptide"

EXHIBIT 2

This is Exhibit 2 referred to in Clause 6 of the Statutory Declaration Siew Yeen Chai dated 13th Day of January 2004.

Before me: 

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THE USE OF ANIMAL MODELS TO STUDY THE EFFECTS OF AGING ON COGNITION

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KEY WORDS: memory, hippocampus, medial temporal lobe, frontal lobe, attention

ABSTRACT

This review addresses the importance of animal models for understanding the effects of normal aging on the brain and cognitive functions. First, studies of laboratory animals can help to distinguish between healthy aging and pathological conditions that may contribute to cognitive decline late in life. Second, research on individual differences in aging, a theme of interest in studies of elderly human beings, can be advanced by the experimental control afforded in the use of animal models. The review offers a neuropsychological framework to compare the effects of aging in human beings, monkeys, and rodents. We consider aging in relation to the role of the medial temporal lobe in memory, the information processing functions of the prefrontal cortex in the strategic use of memory, and the regulation of attention by distributed neural circuitry. We also provide an overview of the neurobiological effects of aging that may account for alterations in psychological functions.

CONTENTS

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INTRODUCTION

From a life-span perspective, the study of aging seeks to understand the changing capacities of the elderly as a normal developmental process. Within this framework, the biology of aging is an important determinant. Just as functions and adaptive capacities depend on the biological development of the young child or adolescent, later life provides a distinctive biological setting in which familiar tasks are performed and new challenges are met. In addition, environmental factors late in life combine with many decades of a person's history to influence the capacities of an individual. We offer the view that important insights into aging as a developmental process can be provided by the study of animal models. The review covers areas of research that illustrate and support this premise.

NORMAL AGING AND ANIMAL MODELS

A major challenge in the study of aging is to define the boundaries of normal change as distinct from pathological conditions. Such boundaries are recognized for development in early life. For example, landmarks are defined for physical growth and cognitive functions; departure from the norm is identified by a failure to manifest certain changes that are expected in the usual course of development. The boundaries of normal aging, as distinct from pathological conditions, are less clearly defined because certain expected changes in cognition during aging do not differ from the earliest manifestations of age-related pathological conditions. For example, the incidence of Alzheimer's disease (AD) increases with advancing age. Although the occurrence of AD is relatively rare before the age of 60, it becomes increasingly prevalent in the decades that follow. Impaired memory, which is the hallmark symptom of AD in its earliest stages, is also one of the most common cognitive features of nondemented elderly individuals (Craik & Jennings 1992, Crook et al 1986). Currently no definitive test can diagnose AD in its earliest stages so that

memory impairment associated with this pathological condition can be separated from memory decline that might be attributable to nonpathological aging.

Age-related neurodegenerative diseases such as Alzheimer's have a slow progressive course. If such diseases remain undetected for many years before a clinically significant phase of decline, relatively subtle changes in presumably healthy older individuals that are ascribed to normal aging might, at least in part, be due to occult pathological processes. Effects of Alzheimer's disease on the brain, such as plaques and neurofibrillary tangles, that meet neuropathological criteria for diagnosis have been detected in autopsy material from individuals not judged to have been impaired by clinical assessment before death (Crystal et al 1988, Morris et al 1991). On this basis, it is likely that studies of elderly human subjects will include some individuals with unrecognized neurological disease.

Additional findings from neurological assessments combined with functional testing support this conjecture. Elderly individuals who have radiological evidence of medial temporal lobe atrophy in the brain along with mild cognitive impairment determined by clinical screening were found to be at high risk for developing dementia (de Leon et al 1993). At follow-up four years after the original assessment, 25 of 86 such subjects had received a diagnosis of AD. Brain atrophic changes are also found in some elderly individuals who show no evidence of cognitive impairment in clinical assessments. In a sample of approximately 150 elderly subjects (ages 55–88 years, mean 70.0 years) who were presumed to be healthy, Golomb et al (1993) found atrophy of the medial temporal lobe in certain individuals. Although no participants in this study showed evidence of cognitive impairment in clinical assessments, differences between the subjects with and without medial temporal lobe atrophy were evident in more sensitive tests of cognitive function. Subjects with atrophy performed more poorly on tests of recent memory than the neurologically normal individuals. Much current interest is also focused on a biological marker, the e4 allele of apolipoprotein E, associated with higher risk for dementia (Corder et al 1993). Recent reports indicate that presumably healthy individuals bearing this marker perform less well on assessments of memory than their aged cohorts (Bondi et al 1995, Helkala et al 1996). Such findings raise the question of whether even mild memory impairment might represent a very early indicator of a pathological process.

Notwithstanding the findings discussed above, other evidence indicates that cognitive alterations in aging occur apart from degenerative neurological disease. Age-associated memory impairment (AAMI) was defined a decade ago (Crook et al 1986) to identify elderly individuals who complain of memory impairment by self-report and have memory test performance at least one

standard deviation below the mean established for young adults. According to these criteria it is estimated that the occurrence of AAMI substantially exceeds what would be expected based on the prevalence and incidence of probable Alzheimer's disease (prevalence and incidence of AAMI about 35% and 6.6% per year compared with 13% and 3% per year for probable AD) (Lane & Snowdon 1989). Furthermore, Youngjohn & Crook (1993) reported that AAMI is stable in elderly individuals based on follow-up assessment four years later. These investigators concluded that AAMI is a relatively benign condition that does not follow a progressive course. Several limitations of this study, however, are worth noting. First, a considerable number of participants that did not return for follow-up testing might have had greater decline than those that did, a phenomenon that is problematic for research of this type. Second, the subjects in this study, who were on average in their early sixties at the first assessment, were followed for a relatively brief interval of four years when the incidence of Alzheimer's disease continues to be low. Further examination of such individuals into later decades might be needed to provide a better indication of the course of AAMI.

A related issue in the definition of normal aging concerns the observation of individual differences in the elderly population. As indicated above, cognitive decline is evident in some individuals in the elderly population (e.g. AAMI), whereas function is better preserved in other aged individuals. In contrast with the usual individual differences that exist at earlier points in development, variability is often described as markedly increased with advancing age. In accordance with this description, a recent survey of published data found greater variability (coefficients of variation) among the elderly compared with young adults on a number of measures widely reported to be sensitive to aging, e.g. reaction time, memory, and fluid intelligence (Morse 1993). Although this phenomenon is often observed, the origin of increased variability in aging is not well defined. Perhaps variability in aging reflects differences that are expressed only in the later decades of life. Alternatively, individual differences might become magnified late in life because of the cumulative impact of different biological and experiential backgrounds over many decades. More information about factors underlying individual differences will be important for understanding normal aging.

The discussion above provides a background for considering the usefulness of animal models in the study of aging. The likelihood that the same pathological processes in human disease occur and are manifest in identical ways across several species could be considered quite low. Many progressive neurological diseases such as Alzheimer's do not afflict species commonly used in laboratory research on aging, e.g. rats, mice, monkeys. However, it is reasonable to expect that at least some features that characterize biological aging of the

mammalian brain would be evident in different species. Thus, commonalities across species might help to identify normal neurobiological aging, as distinct from pathological conditions, and those psychological functions most affected by aging.

The use of laboratory animals can address other aspects of human aging that have proven difficult to study in a systematic way. If normal aging is characterized by increased variability, this phenomenon of individual differences might be evident in other species. Because cohorts of laboratory animals can be maintained under relatively controlled conditions it should also be possible to isolate factors contributing to such individual differences.

The relevance of research with laboratory animals for an understanding of human aging, however, depends on whether the specific functions and biological systems targeted for study are appropriate models for human aging. Scientific advances over the past few decades have provided a foundation for developing useful animal models of aging. We outline a framework currently employed for investigating the neurobiological basis of functional changes in aging. The approach is built on a background of research in neuropsychology, cognitive psychology, and neuroscience.

The field of neuropsychology originally led to clinical descriptions and psychometric profiles for certain types of brain damage as an aid to diagnosis. Because the consequences of certain forms of damage were remarkably selective, neuropsychological studies also came to serve as a basis for making inferences about the normal function of specific brain regions. Alongside developments that came from neuropsychological research, cognitive psychology has greatly contributed to our understanding of psychological processes. Cognitive psychology studies the components and organization of functions such as memory within the framework of information processing and representation. As a related burgeoning field, cognitive neuroscience is building on advances in psychology using new technologies, such as functional neuroimaging and methods for recording the ensemble encoding of information by neurons, to study information processing in the brain. Research derived from these traditions of neuropsychology and cognitive neuroscience has contributed to the development of animal models in the study of aging, as exemplified by the areas covered in the following sections.

MEDIAL TEMPORAL LOBE SYSTEM

Psychological Functions of the Medial Temporal Lobe

Patients with medial temporal lobe damage have circumscribed deficits in memory; the syndrome includes an anterograde amnesia and spares remote memory and general intellectual capacities (Corkin 1984, Scoville & Milner 1957). Anterograde amnesia refers to the inability to remember new informa-

tion and episodes of life that occur after medial temporal lobe damage. The anterograde memory impairment is considered to represent a defect in mechanisms that allow long-term retention of new material. In support of this concept many domains of information processing and immediate memory (e.g. digit span) are preserved in these amnesic patients. Moreover, the recognition that patients with such amnesia have areas of preserved memory, e.g. priming and skill learning, advanced the concept that the brain possesses multiple memory systems (Cohen & Squire 1980). The domain of memory in medial temporal lobe amnesia is variously described as declarative or explicit memory, referring to representations in memory that provide a basis for the conscious recollection of facts and events.

Research using animal models has sought to define the components of the medial temporal lobe that contribute to the amnesic syndrome. Considerable evidence for deficits in declarative memory has been obtained in other species, but agreement has not yet been achieved about the underlying structures subserving memory within the medial temporal system. The most widely used animal model under study in this area of research is a recognition memory task performed by rhesus (or cynomolgous) monkeys (Squire & Zola-Morgan 1991). In this delayed nonmatch-to-sample task, monkeys are presented with an object on an information trial. After a variable delay, the original object is presented with a novel object, and selection of the novel object is rewarded. Considerable consensus surrounds the observation that damage to cortical regions of the medial temporal lobe (perirhinal, entorhinal, parahippocampal cortex) produces a significant delay-dependent deficit in this object-recognition task (Meunier et al 1993, Suzuki et al 1993). Less consensus has been achieved concerning the effects of damage confined to the hippocampus (Murray 1996). However, it is clear that damage to the hippocampus alone produces less severe impairment than damage restricted to the cortical regions of the medial temporal lobe. It is interesting to note that relatively similar findings have been reported in studies of rodents designed to parallel the delayed nonmatch-to-sample task used with monkeys. Damage to cortical structures associated with the hippocampal formation produces delay-dependent impairments that are not observed after selective damage to the hippocampus (Otto & Eichenbaum 1992, Wilner et al 1993).

Different perspectives are offered to account for findings in this line of research. By one view, a common function in memory is served by the component medial temporal lobe cortical regions and the hippocampus, with more severe impairment resulting from more extensive damage to this system (Squire & Zola-Morgan 1991). Another view is that the components of this system may serve somewhat different functions in declarative memory. For example, the relative insensitivity of delayed nonmatch-to-sample tasks to

damage of the hippocampus alone may indicate that the medial temporal cortical regions can subserve memory representation for individual items independent of the hippocampus, while the hippocampus is essential for the formation of more complex representations in memory (Eichenbaum et al 1994). In agreement with this distinction, certain representations that provide a basis for the flexible use of information in memory appear to be highly sensitive to selective hippocampal damage in laboratory animals. For example, it has been argued that spatial information is an exemplar of this type of representation, and it is well documented that severe deficits in spatial tasks are observed after lesions of the hippocampus. Another instance of memory representation sensitive to disruption of the hippocampus comes from a study using probes for memory after animals were trained on a set of nonspatial stimulus-stimulus associations. Normal rats demonstrated two forms of flexible memory that were not shown by rats with selective hippocampal lesions, i.e. transitivity, reflected in the ability to compare across stimulus pairs that share a common element, and symmetry, referring to the ability to associate paired elements presented in the reverse of the training order (Bunsey & Eichenbaum 1996).

Additional research is needed to establish more firmly whether the brain regions that comprise the medial temporal lobe are functionally heterogeneous with respect to their roles in declarative memory. Further advances in this area of cognitive neuroscience will continue to provide an important background for understanding the neurobiological basis of altered memory processes in the elderly.

Psychological Functions of the Medial Temporal Lobe in Normal Aging: Human Beings

The characteristics of memory impairment in presumably healthy elderly adults appear to parallel the general features of medial temporal lobe amnesia (Craik & Jennings 1992). Remote memory and immediate memory (e.g. digit span) are spared. Elderly subjects, however, perform more poorly on typical tests of declarative memory (e.g. paired associates, delayed paragraph recall). Such deficits point to involvement of medial temporal lobe structures. Evidence that alterations in the medial temporal lobe may underlie age-associated memory impairment is noted above; Golomb et al (1993) found that elderly individuals with hippocampal atrophy performed less well on tests of delayed recall.

Functional neuroimaging studies are now providing new information about the relative activation of this system in young and elderly individuals during performance of memory tasks. Grady et al (1995) measured cerebral blood flow during encoding and recognition of faces. They reported that poorer memory performance in healthy elderly individuals relative to young individuals was associated with a reduction in hippocampal and prefrontal cortical

activation during encoding. In the context of this observation, it is noteworthy that hypoactivity in the medial temporal lobe is not invariably observed in the brains of elderly subjects. In contrast with the results of Grady et al, another recent study found comparable medial temporal lobe activation in young and elderly subjects during successful recall (Schacter et al 1996). In that experiment, a word-stem completion task was administered to produce either high or low recall of study words. When neuroimaging was done during retrieval tests, equivalent hippocampal activation in young and aged groups was observed during successful recollection (high recall versus either low recall or baseline), which indicates that an age-related deficiency localized to the medial temporal lobe does not occur in all conditions where hippocampal activation is observed. These results also suggest that memory deficits in the elderly are not due to deficient medial temporal function during the retrieval of information. Rather, deficits may be attributable, at least in part, to a functional impairment in medial temporal lobe processing of new information, i.e. encoding, that serves as a basis for later recognition or recall.

Before turning to the examination of memory performance in aged laboratory animals, we reiterate that age-associated memory impairment is not evident in all individuals in the elderly human population. Thus, it is of interest in studies of laboratory animals to assess whether similarities can be found in the effects of aging on memory that resemble those features encountered in human beings and to determine whether individual differences also exist in other species over the course of aging.

Psychological Functions of the Medial Temporal Lobe in Normal Aging: Animal Models

The delayed nonmatching-to-sample task used to assess recognition memory in young monkeys with medial temporal lobe damage has also been used to test aged monkeys (Moss et al 1988, Presty et al 1987, Rapp & Amaral 1989). Although aged monkeys have difficulty in learning this task with a very brief retention interval, given sufficient training virtually all older subjects are able to reach a criterion equivalent to young monkeys. When the memory demands are then manipulated by increasing delays, monkeys approximately 25 years or older are impaired. Individual differences in recognition memory among aged monkeys are also observed, with an impairment in a subset of aged monkeys that qualitatively resembles the effect of medial temporal lobe damage in young monkeys (Rapp & Amaral 1991). Furthermore, aged monkeys with such deficits also perform more poorly on rapidly learned two-choice object discrimination problems, another assessment that is sensitive to medial temporal lobe damage (Rapp 1993). An additional parallel in the pattern of impairment across aged monkeys and young monkeys with medial temporal lobe damage is found in a task that increases the load of information in memory.

Subjects are tested for identification of each new item added to an array of previously presented items (Killiany et al 1995). Thus, studies of nonhuman primates have demonstrated memory impairments on tests sensitive to the integrity of the medial temporal lobe. Moreover, the presence and/or severity of such deficits varies considerably in the aged population. Because the neural substrate within the medial temporal lobe system for the most commonly used task in this research, delayed nonmatching-to-sample, is not clearly defined, age-related impairment on this assessment might reflect alterations in medial temporal lobe cortical systems either alone or together with alterations in the hippocampus.

Other evidence for alterations in the hippocampus in aged monkeys comes from a report of impairment in the flexible use of information in memory. As noted in the prior section, a test for the use of information in memory that is sensitive to selective damage of the hippocampus has recently been demonstrated by probes for transitive inference in rodents. After learning a set of stimulus-stimulus associations, young intact rats infer relations among the items, an ability that is lacking in rats with lesions confined to the hippocampus (Bunsey & Eichenbaum 1996). In a recent study, monkeys that learned a hierarchy of object-object discriminations were tested for their use of information in memory. The performance of aged monkeys during probes failed to show response latency effects that are taken to reflect the relational processing of information that underlies transitive inference (Rapp et al 1996).

Deficits that are widely studied in aged rodents in spatial tasks may also reflect a declarative memory impairment. Aged rats, like young rats with damage to the hippocampus, have deficits in a variety of spatial tasks (for an overview, see Gallagher et al 1995). Moreover, impaired spatial learning in aged rats or in young rats with hippocampal damage can be demonstrated to occur independent of decline in sensorimotor/motivational functions and in learning that is guided by a stimulus or object used as a local cue (Gage et al 1989, Gallagher et al 1993). In addition, individual differences have been particularly well documented in this line of research. Among certain strains of rats, a proportion of aged animals exhibit highly preserved performance on such tasks, while other aged cohorts perform entirely outside the range of young rats (Gallagher et al 1993).

With respect to the neurological basis for impairments of aged rodents on spatial tasks, note that these tests do not provide an entirely selective assessment of the function of the hippocampus (Gage et al 1984). For example, young rats with lesions of cortical systems interconnected with hippocampus, e.g. entorhinal/perirhinal cortex, also exhibit deficits in spatial tasks (Nagahara et al 1995). Thus additional assessment is needed to distinguish between impairment that may have a basis in the altered status of the hippocampus

versus these cortical regions. As noted earlier, a version of delayed nonmatch-to-sample developed for studies of rodents has revealed a sensitivity to entorhinal/perirhinal cortex damage that was not seen after selective damage to the hippocampus in young rats (Otto & Eichenbaum 1992). A recent study reported that aged rats that learned this task at short retention intervals performed no differently than young rats when increasing delays were introduced (Zyzak et al 1995). The same rats used in this assessment were also evaluated in a spatial task where an age-related deficit was observed. Thus, aged rats that are impaired in a spatial task that is sensitive to the integrity of the hippocampus can display intact performance on an assessment that is more selectively sensitive to the integrity of related cortical regions in the temporal lobe. Such results support the concept that impairment in spatial tasks reflects an effect of aging on the hippocampus.

Behavioral studies demonstrating a decline in functions associated with medial temporal lobe structures in laboratory animals provide evidence for age-associated memory impairment independent of pathological conditions that affect the elderly human population. Moreover, individual differences in aging are often documented in studies of nonhuman primates and rodents, which provides an additional parallel with observations in human beings.

Neurobiology of Aging in the Medial Temporal Lobe

Much current research in laboratory animals is directed at the neurobiological basis of decline in cognitive functions associated with the medial temporal lobe. Two concepts about the basis for this decline in aging, which have prevailed for several decades, have recently come under new scrutiny. In the first case, neurodegeneration within the hippocampus had been thought to play a significant role (Meaney et al 1988). A second influential concept held that degeneration in the basal forebrain cholinergic system, a component of which innervates the hippocampus and related cortical structures, provides a basis for memory deficits in aging (Bartus et al 1982). In each case, recent studies indicate that these long-standing concepts may be incorrect. After discussing the research dealing with those topics, we consider other potential substrates for age-related loss of function in the medial temporal lobe system.

The conclusion that neuron loss occurs in the aged hippocampus was reached in earlier studies of human, nonhuman primate, and rodent brains (Brizzee et al 1980, Dam 1979, Issa et al 1990, Meaney et al 1988). In some of these reports, the concept that neurodegeneration causes age-related cognitive decline was further bolstered by evidence that the presence and severity of behavioral impairment were correlated with loss of principal neurons in the hippocampus (Issa et al 1990). It is important to note, however, that studies of neurodegeneration were originally based on methods for estimating neuron density. Neurodegeneration is now being studied using new methods that are

unbiased for many factors that could influence measures of neuron density, such as the size of neurons and the size or composition of nonneural cells, and so forth. Recent results using these new methods for measuring the total number of neurons in a brain structure indicate that no neuron loss appears to occur in the hippocampus during normal aging across a variety of species.

Studies of aging in human, nonhuman primate, and rat brain have now shown equivalent numbers of the principal neurons (Figure 1) in the hippocampus (Rapp & Gallagher 1996, Rasmussen et al 1996, West 1993, West et al 1993). Moreover, the recent anatomical studies of rats showed that even aged animals with substantial deficits indicating hippocampal dysfunction exhibited no loss of neurons in this structure. For example, Rapp & Gallagher (1996) observed a wide range of spatial ability in the aged rats included in their study but relatively little variability in numbers of hippocampal neurons and no suggestion of a decline in neuron number associated with age or behavioral impairment.

Additional evidence indicates that neuron number is preserved in the hippocampal formation of aged nonhuman primates, including those monkeys with identified memory impairment (West et al 1993). In addition to comparable numbers of principal neurons in the hippocampus proper, this study found no significant loss of neurons in cortical regions associated with the hippocampal formation (e.g. entorhinal cortex and subiculum). This finding is particularly noteworthy because a subset of the aged monkeys had deficits in recognition memory assessed in delayed nonmatch-to-sample, a task that is sensitive to the integrity of cortical areas in the medial temporal lobe.

Neurodegeneration in the hippocampus was formerly viewed as an inevitable consequence of normal aging. Reports that the principal neurons of the hippocampus are preserved across a variety of species, even in the presence of substantial behavioral impairment, may prompt a shift in the view that neuronal loss in this structure serves as a basis for age-related cognitive decline. Neurodegeneration in the hippocampus also appears to distinguish certain pathological conditions from normal aging. In contrast with normal aging, stereological methods detect significant reductions in the number of principal neurons in the hippocampus in individuals with diagnosed Alzheimer's disease (West et al 1994).

A second long-standing concept about the neurobiological basis of cognitive decline in aging has focused on the basal forebrain cholinergic system (Bartus et al 1982). Cholinergic neurons within this system that are located in the medial septum and vertical limb of the diagonal band (MS/vDB) provide innervation of the hippocampus and related medial temporal cortex (Koliatsos et al 1990). Atrophy and degeneration of these neurons is detected in aged brains (Fischer et al 1989, Smith & Booze 1995, Stroessner-Johnson et al 1992), and marked pathology affects this system in Alzheimer's disease

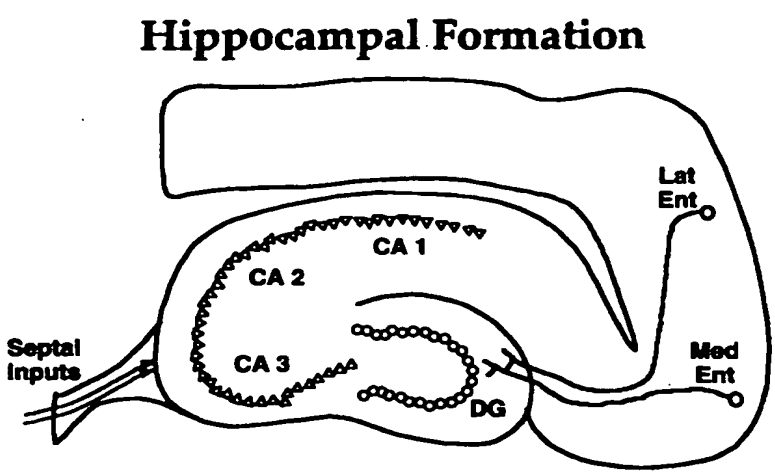

Figure 1 The schematic shows the principal neurons of the hippocampal formation, the granule cells of the dentate gyrus (DG), and the pyramidal neurons of the hippocampus proper (areas CA1–CA3). Two major input pathways to the hippocampal formation are illustrated. Input from the lateral and medial entorhinal cortex (Lat Ent and Med Ent) provides highly processed information and terminates primarily on the dendrites of the granule cells in the dentate gyrus. A subcortical pathway (Septal Inputs) enters through another route and provides widespread innervation of the hippocampal formation. This latter input includes the cholinergic innervation of the hippocampal formation. A trisynaptic circuit through the hippocampus (not shown) begins with the synapses formed by the entorhinal cortex projection onto the dentate granule cells. Those cells in turn project to the CA3 area. The CA3 pyramidal neurons form a third set of synapses within this structure, projecting onto the pyramidal cells in the CA1 area.

(Coyle et al 1983, Davies & Maloney 1976). It is further noteworthy that the number and size of these neurons are affected by age across a variety of species, including rats, monkeys, and human beings. The possibility that neurodegeneration within this population of neurons contributes to functional impairment has found support in numerous studies showing that the amount of cholinergic neuron deterioration is related to the severity of behavioral deficit in aged subjects (for an overview, see Gallagher et al 1995). Note that the vast majority of these correlational studies have used rat performance on spatial tasks as the behavioral assessment. Recent evidence, however, challenges the conclusion that deterioration of cholinergic neurons can account for age-related impairments in spatial tasks (Gallagher & Colombo 1995). At issue in this line of research is not whether degeneration occurs within the basal forebrain cholinergic system, but whether that effect of aging causes behavioral decline.

One way to assess the contribution of the septohippocampal cholinergic system to age-related impairments is to examine whether removing these

neurons in young animals reproduces the deficits observed in aging. A newly developed immunotoxin 192 IgG-saporin can be used to target selectively basal forebrain cholinergic neurons. After injection of the immunotoxin into the MS/vDB, a nearly complete removal of the cholinergic innervation of the hippocampus can be achieved. It has come as a surprise, given the cholinergic hypothesis of age-related impairment, that immunotoxin-induced lesions of the septohippocampal cholinergic system fail to produce reliable spatial learning deficits (Baxter et al 1995a, 1996; Berger-Sweeney et al 1994, Torres et al 1994). Young rats with over 90% depletion of the cholinergic-specific enzyme choline acetyltransferase (ChAT) in hippocampus perform normally on a spatial learning protocol that is highly sensitive to deficits in aged rats (Baxter et al 1995a), and no impairment is even detected after removal of the entire basal forebrain cholinergic system, including the input to the hippocampal formation and the widespread innervation of the cerebral cortex (Baxter et al 1996). Moreover, comparable cholinergic lesions in aged rats do not appear to exacerbate or induce impairment in spatial tasks (Baxter & Gallagher 1996). Thus it is unlikely that deterioration in the septohippocampal cholinergic system by itself provides a sufficient basis for age-related deficits that are commonly observed in spatial tasks. We return in a later section of this review to the function of the cholinergic innervation of the hippocampus and its possible contribution to behavioral deficits in aging.

A current theme in research on normal aging is that a reduction in the number of synaptic connections, rather than frank neurodegeneration, provides a basis for age-related alterations in cognition. For example, atrophy and degeneration in the basal forebrain cholinergic system is likely to result in some loss of hippocampal innervation by these neurons. In agreement with this expectation, the cholinergic response mediated by stimulation of the septohippocampal input is reduced in all areas of the aged rat hippocampal formation (Shen & Barnes 1996). However, the failure of specific cholinergic lesions to reproduce memory deficits that have been well documented in aged rodents makes it unlikely that this alteration, by itself, serves a broad basis for impairments in aged rats.

Apart from the subcortical input to the hippocampus that originates in the septal region, an additional loss of synaptic input from another source is well documented to occur in aging. That input, which provides the primary route for transfer of highly processed cortical information to the hippocampus, derives from neurons in the entorhinal cortex (refer to Figure 1). Ultrastructural studies of rat brain have demonstrated a significant loss of synaptic connections in the hippocampal formation that are formed by entorhinal cortex input (Geinisman et al 1992). In addition, individual differences in cognitive decline among aged rats are reported to coincide with differences in the loss of this

innervation (Geinisman et al 1986). Reduced numbers of synaptic connections may also occur in other areas of the hippocampal formation (Barnes et al 1994).

It might be reasonable to expect that reduced numbers of synaptic connections in the hippocampal formation could provide a basis for behavioral deficits that depend on the integrity of this system. However, it should be recognized that a number of factors might argue against that outcome. Neurobiological systems possess a number of mechanisms that are geared to maintain function. Considerable evidence indicates that such mechanisms are recruited in the aged brain. For example, although fewer synaptic connections are made in the dentate gyrus of the hippocampal formation in aged rats, the response to input at the remaining synapses is greatly increased (Foster et al 1991). In addition, a loss of synapses from cortical input induces a sprouting response in which new connections are formed by inputs from other sources. Evidence for such sprouting in the hippocampal dentate gyrus has come from studies of aged brains and is also observed in Alzheimer's disease (Geddes et al 1992, Nicolle et al 1996). This type of sprouting may be compensatory in nature, as new connections are made to replace those that are lost. However, such reactive growth may not always be beneficial. New connections might add to the adverse effects of aging because they come from a different source than the original input. Studies of aging often lack the functional analysis necessary to distinguish between these possibilities. Recent studies using behavioral assessment along with neurobiological analysis are being conducted to evaluate whether such alterations in the brain provide protection or impose further adverse effects on outcome (Nicolle et al 1996, Stack et al 1995, Stenvers et al 1996). If a neurobiological response in the aged brain is compensatory, then the degree to which this response occurs should predict a better outcome, i.e. less behavioral impairment. However, if a reactive process only adds to dysfunction then the presence of such a change in the brain might be associated with greater impairment. Research of this type should lead to a better understanding of the consequences of reactive and reorganizational processes that occur in the aged brain.

Studies of neurodegeneration and synaptic connectivity provide important information about structural features of the brain during aging. Apart from such structural features, effects of aging are evident in the functional integrity of existing neurons and connections. Such changes may be important factors in diminishing the overall performance of the medial temporal lobe system during aging.

Neurons in the hippocampal system possess a complex array of biological mechanisms for information processing. In addition to specialized receptors for specific inputs, receptors are coupled to a variety of transduction systems

that are necessary to produce physiological responses to those inputs (refer to Figure 2). Furthermore, transduction mechanisms are not only used for the processing of information transmitted between neurons but also play a role in altering the functional properties of synaptic connections. Long-term potentiation (LTP), referring to the long-lasting increase in the effectiveness of synaptic connections that can be readily induced in the hippocampal system, has attracted widespread interest as a possible physiological mechanism for the storage of information in the mammalian brain.

Studies on transduction mechanisms may provide insight into the basis for changes in information processing and information storage during aging. The findings from such research are especially informative in a system where neurodegeneration is not a prominent feature of aging. In such a setting, measurable decreases in components of transduction systems, including receptors, coupling mechanisms, and second and third messengers, do not merely reflect a loss of neurons but indicate a change in the functional integrity of existing neurons. For this reason, recent evidence for preserved numbers of

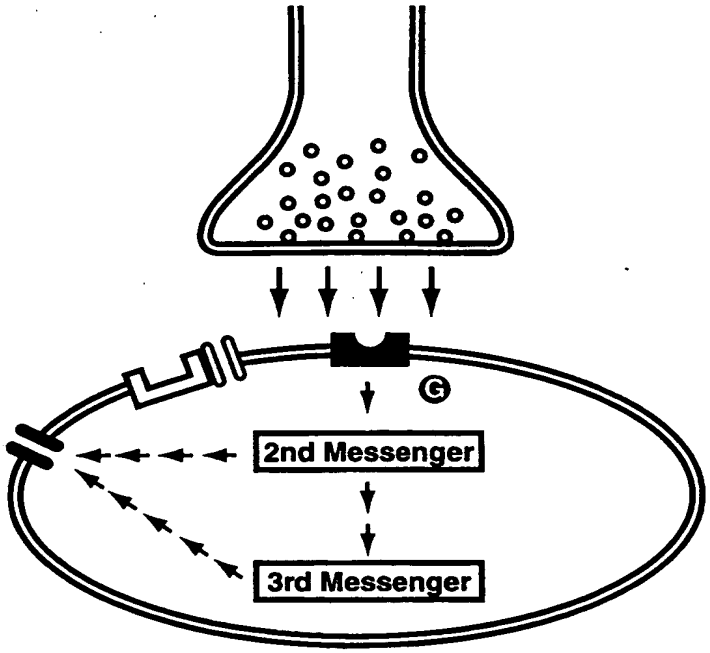

Figure 2 Schematic illustrates interneuron communication. Transmission from an input produces a postsynaptic response through a variety of transduction mechanisms. The components of this communication system include receptors that either directly regulate a neuron's excitability or work through biochemical cascades (2nd and 3rd messenger systems).

neurons in the hippocampus is important for interpreting the effects of aging on other neurobiological measures.

In addition to an absence of frank neurodegeneration within the hippocampus during aging, at least some types of receptors that serve as targets for neurotransmitters are also relatively unaffected by aging in this structure. For example, substantial preservation of postsynaptic receptors for acetylcholine is reported in a number of studies (Chouinard et al 1995, Quirion et al 1995, Smith et al 1995). To the extent that postsynaptic targets are preserved, then strategies to address the effects of aging might be developed to increase function at these sites. Such a rationale has served as a basis for developing drugs to compensate for age-related deterioration of the basal forebrain cholinergic neurons. Acetylcholinesterase inhibitors (e.g. Tacrine) are intended to augment the action of acetylcholine at its receptors in the forebrain by preventing the degradation of this transmitter (Davis et al 1992, Thal et al 1989). Because such drug treatments can improve cognitive function in young rats, some benefit might be achieved in aged animals even if the basis for impairment is not solely due to a cholinergic defect (Gallagher & Colombo 1995). The effectiveness of augmenting cholinergic function would depend, however, on an intact physiological response at cholinergic receptors. In the case of those receptors, however, a blunted response to cholinergic stimulation has recently been documented in the aged rodent hippocampus (Chouinard et al 1995, Undie et al 1995). In one of these studies, a greater reduction in the postsynaptic response to cholinergic stimulation was seen in aged rats that were found to have more pronounced cognitive impairment (Chouinard et al 1995). Moreover, the neurobiological defect in this case appeared to reside at a point in the biochemical machinery that would potentially affect the physiological response to other transmitter/receptor inputs that use the same transduction pathway.

In addition to the role that transduction mechanisms serve in information processing, long-lasting changes in the properties of synapses depend on the functional integrity of neurons. It has long been recognized that alterations in the mechanisms required for neural plasticity could provide a basis for cognitive decline during aging. One of the earliest studies of individual differences in spatial learning in aged rats showed that this impairment correlated with a deficiency in neural plasticity in hippocampus (Barnes 1979). In those experiments an in vivo study of LTP at perforant/dentate synapses was conducted in the same rats that were behaviorally tested in a spatial learning task. Although asymptotic (saturated) LTP did not differ between the age groups, this LTP was achieved less readily and decayed more rapidly in the aged rats than in young rats. Furthermore, the impairment in behavioral learning was significantly correlated with the effect of aging on LTP. Additional studies continue to document that LTP is adversely affected during the aging process (Barnes &

McNaughton 1985, Moore et al 1993). A modest loss of the receptors required for induction of LTP may occur in the aged hippocampus in both primates and rodents (Clark et al 1992, Gazzaley et al 1996, Nicolle et al 1996, Wang et al 1996). A highly active area of research aimed at defining the mechanisms underlying LTP in adult animals will provide a route for better understanding the basis of the deterioration seen in aging.

Consistent with a variety of evidence for functional alterations in the aged hippocampal formation, recording the activity of hippocampal neurons while animals perform certain tasks has provided evidence that the representation of information in this system is altered in older animals. Such studies have shown less reliability and specificity of the information encoded by hippocampal neurons (Barnes et al 1983, Mizumori et al 1996; but see Markus et al 1994). In a recent study of aged rats, individual differences were also evident. In aged rats that were impaired in a cognitive assessment of spatial learning, representations of relationships among stimuli in a spatial environment were relatively impoverished and inflexible compared with either young rats or aged cohorts with preserved behavioral functions (Tanila et al 1996). Further research of this type will help to elucidate the computational cost of the neurobiological effects of aging within this brain system. Identification of mechanisms underlying diminished function within that circuitry will undoubtedly provide an impetus to the development of new therapeutic strategies to treat age-related impairment.

In conclusion, cognitive impairments that resemble those seen in elderly human beings can be observed in the study of aged laboratory animals. Individual differences in the effects of aging on tasks sensitive to the integrity of the medial temporal lobe are also mirrored in the presence and severity of some neurobiological changes found in this system. Beyond the effort to construct a description of normal aging, research using animal models promises to provide a setting for productive research on mechanisms of brain aging. This may include a better understanding of how the rate or severity of aging provides a basis for individual differences in cognitive abilities late in life.

FRONTAL LOBE SYSTEMS

Psychological Functions of Frontal Lobe Systems

In contrast with amnesia resulting from medial temporal lobe damage, human beings with frontal lobe lesions perform accurately on many standard tests of declarative memory (reviewed in Moscovitch & Ulmita 1991). Current perspectives instead emphasize that the prefrontal cortex supports a variety of organizational processes that importantly influence the strategic use of memory. In addition, compelling evidence has revealed functional heterogeneity

across the regions comprising the prefrontal cortex. A popular view is that these areas function in a "central executive" capacity, mediating the on-line manipulation of memory, particularly under circumstances emphasizing the spatial, temporal, or other contextual attributes of acquired information (reviewed in Moscovitch & Ulmita 1991). Recent evidence consistent with this view comes from neuroimaging studies in normal human subjects. Cerebral blood flow is selectively increased in a region of the dorsolateral prefrontal cortex when memory for temporal order is necessary for successful performance relative to conditions involving the same sensory and motor demands but lacking a temporal order component (Petrides et al 1993a,b). A slightly more posterior prefrontal region (area 8), by comparison, is activated during a conditional discrimination procedure placing relatively greater emphasis on the environmental contingencies governing ongoing behavior (Petrides et al 1993a). Such data support the concept that the prefrontal cortex comprises a variety of functionally distinct subsystems. In addition, this background of information helps to account for the pattern of impairments observed following frontal lobe damage, which includes prominent deficits in memory for temporal order, impaired recall for the source of acquired information (i.e. source amnesia), and difficulties modifying behavior appropriately in response to changing environmental contingencies (i.e. perseveration) (Janowsky et al 1989a,b; Shimamura et al 1990).

Another feature of specialization within the prefrontal cortex is suggested by studies focusing on the component processes of declarative memory, e.g. encoding, retrieval, and so forth. As noted previously, encoding processes and the successful conscious recollection of events are associated with hippocampal activation (Grady et al 1995, Schacter et al 1996). Lateralized prefrontal cortical activation is particularly associated with effortful retrieval of information from memory (Schacter et al 1996). This observation supports the concept that the activity of prefrontal cortex is engaged by specific retrieval strategies in support of declarative memory (Buckner & Petersen 1996). By this account, successful performance on tests of declarative memory in patients with frontal damage presumably reflects the utilization of alternate retrieval mechanisms mediated by intact structures. Findings from studies of normal human aging, reviewed in the next section, are consistent with this proposal.

Psychological Functions of Frontal Lobe Systems in Normal Aging: Human Beings

Although deficits in declarative memory are frequently observed in the elderly, older subjects exhibit a variety of impairments that would not be anticipated as a consequence of dysfunction restricted to the medial temporal lobe. Many of the most prominent and consistent signs of age-related cognitive decline in-

stead occur in the information-processing capacities traditionally associated with the prefrontal cortex (for recent reviews, see Rapp & Heindel 1994, Shimamura 1994). For example, normal elderly individuals have difficulty remembering the source of acquired information (Janowsky et al 1989b, McIntyre & Craik 1987, Naveh-Benjamin & Craik 1995), even under circumstances where explicit recollection of target items is relatively intact (Dywan et al 1994, Glisky et al 1995). Source memory deficits can also predict performance on other tests of frontal lobe function, which suggests that a common neurobiological basis may underlie these impairments (Craik et al 1990, Glisky et al 1995; but see Spencer & Raz 1994). A further parallel with the effects of frank frontal lobe damage is that memory for temporal order appears particularly susceptible to decline as human beings age (Daigneault & Braun 1993, Parkin et al 1995).

It is noteworthy that certain impairments associated with the function of prefrontal cortex emerge relatively early in the life span, during middle-age, and are unrelated to the status of encoding and retrieval processes that support normal declarative memory. Moreover, when age-related impairments that resemble both medial temporal and prefrontal dysfunction coexist in the same persons, these cognitive deficits may be somewhat dissociable. A particularly interesting report relevant to this point studied healthy aged individuals between the ages of 65 and 87 years (Glisky et al 1995). Two factors were obtained in a factor analysis of the neuropsychological test data. Tests traditionally viewed as assessing the status of prefrontal cortex (e.g. Wisconsin Card Sorting) loaded onto one factor, whereas assessments of declarative memory sensitive to medial temporal lobe status (e.g. paired associates, delayed cued recall) loaded strongly onto a second factor. A subsequent study of item and source memory showed a double dissociation among these elderly individuals that corresponded with their relative functioning on medial temporal lobe and prefrontal assessments, respectively. In addition to suggesting that there is not necessarily an obligatory relationship in the effects of aging across different information processing domains, these data suggest that the underlying biological alterations that cause decline in medial temporal lobe- and prefrontal cortex–dependent functions may occur somewhat independently.

In agreement with the neuropsychological assessments of aged human beings noted above, assessment of the neurobiological status of the prefrontal cortex indicates its susceptibility to age-related decline. Cortical atrophy during normal human aging is especially pronounced in the frontal lobe, progressing at a rate greatly exceeding atrophy observed in the cerebral hemispheres as a whole (Coffey et al 1992). Measurements of regional cerebral blood flow under a variety of testing conditions also provide an indication of diminished function. One of the principal findings to emerge from this approach is that

task demands sufficient to produce prefrontal cortical activation in young subjects fail to increase activity in this same region in older adults (Grady et al 1995). Neuroimaging has also localized different patterns of activation coincident with efforts to retrieve information, relative to activity induced by the conscious recollection of target items (Schacter et al 1996). In young subjects, retrieval efforts are accompanied by significant activation in anterior aspects of the frontal lobe, but a more posterior frontal region is activated in aged subjects under the same testing conditions. No age difference, in contrast, was observed during the successful recollection of target information, which predominantly engages medial temporal lobe structures. The interesting implication of these results is that when recollection is not readily achieved, young and aged subjects may use different retrieval strategies, mediated by distinct prefrontal processing systems. Independent of the validity of this particular hypothesis, it is evident that abnormalities in the activation of prefrontal cortex occur in relation to cognitive aging.

Psychological Functions of Frontal Lobe Systems in Normal Aging: Animal Models

The development of a nonhuman primate model of normal cognitive aging has revealed a number of interesting parallels with findings in human beings. Deficits on delayed response tests of short-term memory are among the most conspicuous and well characterized signs of behavioral decline in the aged monkey (Bartus et al 1978, Dean & Bartus 1988). In the standard delayed response task, a reward, placed in one of two locations, is retrieved by the monkey after a varying delay. There is compelling evidence, however, that the delayed response deficit is not necessarily symptomatic of a general memory impairment of the type that results from damage to the medial temporal lobe. For example, aged monkeys with pronounced delayed response deficits often perform normally on standard tests of recognition memory (i.e. delayed nonmatch-to-sample) and on a variety of other procedures that are sensitive to medial temporal lobe lesions (Bachevalier et al 1991, Rapp & Amaral 1989). In addition, delayed response impairments emerge relatively early in the life span, preceding the decline in memory abilities that require the functional integrity of the medial temporal lobe (Bachevalier et al 1991). Consistent with conclusions from human research discussed in the prior section (Glisky et al 1995), these findings emphasize that age effects are not uniform across different information-processing domains, and that medial temporal lobe dysfunction alone may fail to account for certain key features of cognitive aging in nonhuman primates (for recent reviews, see Dean & Bartus 1988, Rapp 1995).

A number of the behavioral impairments observed in aged monkeys appear to reflect a decline in memory-related processes mediated by the prefrontal

cortex (Dean & Bartus 1988, Rapp 1995). In this context, a noteworthy aspect of standard delayed response testing is that it makes substantial demands on memory for temporal order. This is a consequence of the procedural arrangement in which a reward is hidden randomly, across trials, among a relatively small number of possible locations. Accurate performance therefore requires memory for the location baited most recently, and the ability to discriminate the current trial from information presented earlier in testing. Standard delayed response testing also incorporates an explicit spatial component that is thought to specifically engage processing functions of the dorsolateral prefrontal cortex (Wilson et al 1993). Consistent with the view that delayed response deficits reflect prefrontal cortical decline, aged monkeys exhibit deficits on other tasks as a function of demands on temporal ordering (Rapp & Amaral 1989). Increased perseveration is also observed in aged nonhuman primates (Anderson et al 1993, Bartus et al 1979), similar to effects seen in aged human beings (Janowsky et al 1989b), and qualitatively resembling the difficulties young subjects with frontal lobe damage display in modifying behavior under conditions of shifting task contingencies (Janowsky et al 1989a).

A unified perspective on the functional organization of the prefrontal cortex that accommodates results from both rats and primates has yet to be achieved. Nonetheless, studies of aged rats have noted a number of qualitative similarities with the behavioral effects of frontal lobe damage in young adult rats. In a direct comparison of this type, Winocur (1992) evaluated delayed nonmatch-to-sample performance in young and aged groups, and in young rats with lesions of either the prefrontal cortex or dorsal hippocampus. The sample stimulus in this operant procedure consisted of a panel light that was illuminated at one of two intensities. During the recognition phase of each trial, reward was contingent on the rat's committing or withholding a lever response (i.e. "go," "no-go") depending on whether a matching or nonmatching light intensity was presented. Similar to delayed response testing in monkeys, the opportunity for intertrial interference is substantial in this procedure, and successful performance requires animals to distinguish between the current sample and the same items presented in earlier trials. Aged rats, and young rats with prefrontal cortical lesions, displayed substantial acquisition deficits under conditions where no delay was imposed between the sample presentation and recognition test. In contrast, young rats with hippocampal lesions acquired the task at a normal rate. These findings broadly parallel results in monkeys and human beings, consistent with the view that the temporal organization of memory is significantly disrupted in the aged rat. Qualitative similarities in the effects of aging and direct prefrontal cortical damage have also been noted in studies using other behavioral testing procedures (Winocur 1991, Zyzak et al 1995).

Neurobiology of Aging in Frontal Lobe Systems

Compared with the research on the medial temporal lobe system reviewed above, only limited experimental attention has focused on defining the neurobiological consequences of prefrontal cortical aging. Recent neuroimaging, however, has revealed that metabolic activity in the monkey prefrontal cortex declines with age, and interestingly, that variability among aged subjects is substantially greater than among young animals (Eberling et al 1995). On this basis, it is tempting to speculate that individual differences in metabolic activity might predict the status of cognitive processes mediated by the prefrontal cortex. Preliminary findings from a study combining functional neuroimaging and delayed response assessment in the same subjects suggest that this may be the case (Roberts et al 1996).

Changes in the structural integrity of the prefrontal cortex are currently under examination as a possible basis for age-related cognitive decline. Consistent with a growing body of evidence indicating that neuron number is generally preserved during normal aging in the medial temporal lobe cortical structures (Rapp & Gallagher 1996, Rasmussen et al 1996, West 1993, West et al 1993; and see section on "Neurobiology of Aging in the Medial-Temporal Lobe"), Peters et al (1994) failed to observe any age-related decline in neuron density in the dorsolateral prefrontal cortex. A subjective scoring of white matter pathology in the same subjects, however, revealed prominent age effects, with the greatest degree of change apparently observed among aged monkeys that were most impaired on standard tests of learning and memory. Subtle age-related alterations in other morphological parameters have also been noted, including a decline in the dendritic arborization of prefrontal cortical neurons (Cupp & Uemura 1980). Providing an additional parallel with studies of the medial temporal lobe, structural features in the prefrontal cortex are relatively intact, with possibly greater alterations in neuropil and connectivity as opposed to frank neurodegeneration of cortical neurons.

Compared with the relatively preserved structural features of prefrontal cortex, considerable evidence points to a substantial impact of age on subcortical systems that project to cortex. In addition to the cholinergic neurons in the basal forebrain system, several collections of monoamine neurons in the brainstem appear to undergo significant degeneration and/or atrophy during aging (DeKeyser et al 1990, Irwin et al 1994). In young subjects, systemic pharmacological manipulations of noradrenergic and dopaminergic function significantly influence spatial and temporal aspects of memory, and at least some of these effects appear to be mediated at the level of cortical projection targets in the frontal lobe (Murphy et al 1996). These normative findings, then, lead to the expectation that age-related alterations in neurochemically defined subcor-

tical projection systems might significantly disrupt information processing functions dependent on the prefrontal cortex.

Concerning the function of prefrontal cortex, considerable evidence indicates that age-related alterations in its dopaminergic innervation may be particularly important. This interpretation is consistent with electrophysiological results demonstrating that application of dopamine receptor antagonists can modulate the memory-related firing properties of single prefrontal cortical neurons (Williams & Goldman-Rakic 1995). Research addressing the neurochemistry of aging in the monkey indicates that endogenous dopamine concentrations are markedly reduced in the prefrontal cortex and that this decline is substantially greater than that observed in other cortical regions (Goldman-Rakic & Brown 1981, Wenk et al 1989). In addition, Luine et al (1990) observed that during aging in the rat, a dopamine deficiency in the frontal cortex was significantly correlated with impaired working memory performance on a radial maze. Although dopamine agonist administration in young subjects can affect a variety of behavioral domains including motor function, effects of dopaminergic agents in aged monkeys are selectively attenuated on tasks that require the functional integrity of the prefrontal cortex, such as the standard delayed response task (Arnsten et al 1995). Combined with a substantial body of earlier research (reviewed in Arnsten 1993), these findings raise the possibility that alterations in subcortical systems, such as the mesocortical dopaminergic neurons, might contribute to certain aspects of cognitive aging by disrupting the information processing functions of cortical target regions in the frontal lobe. This area of research also supports the broader theme, developed throughout this review, that cortical and subcortical brain systems are differentially sensitive to the neurobiological consequences of normal aging. The neurodegeneration often associated with brain aging appears to be more characteristic of certain subcortical systems that innervate forebrain structures than of cortical neurons themselves.

AGING AND ATTENTION IN HUMAN BEINGS AND ANIMAL MODELS

As noted in the preceding sections, certain effects of aging on cognition resemble, in mild form, damage to systems in the forebrain, including the medial temporal lobe and prefrontal cortex, for which a substantial background of neuropsychological research exists. Furthermore, neurobiological studies are beginning to provide an understanding of alterations in the brain that are most likely to serve as a basis for cognitive decline in functions associated with those systems. Alongside these areas of research, interest in the study of attention in aging has grown in recent years.

Attention refers to multiple component functions that are important in the selection and processing of information. The study of attention in aging is currently benefiting from advances in cognitive neuroscience that are providing a better definition of the neural systems that are critical for the normal regulation of attention. For example, these include systems that regulate overall levels of sustained attention (arousal or vigilance) and systems that are important for the selective processing of information among competing inputs. Sustained attention can be assessed in settings that require performance of a simple task without the subject losing track of the task objective, a function that appears to be little affected by aging (Albert & Moss 1996). In contrast, other evidence points to an effect of aging on the selective processing of information, particularly under conditions of competition among many items for processing resources (Greenwood et al 1993, Mouloua & Parasuraman 1995).

As recounted in the chapter in this volume on "Central Cholinergic Systems and Cognition" (Everitt & Robbins 1997), a role in the regulation of attention may represent the primary function of the basal forebrain cholinergic neurons that innervate cortex. Furthermore, a growing consensus now views the neurodegeneration within this system that occurs in aging, and to a more severe degree in Alzheimer's disease, as providing a basis for deficits in attention rather than underlying a decline in memory processes (Parasuraman & Haxby 1993).

The cholinergic neurons in the basal forebrain that provide widespread innervation of the cortex in rats, monkeys, and human beings are located posterior to the cholinergic neurons in the basal forebrain that target the hippocampal formation (Koliatsos et al 1990). Previous studies have revealed deficits in attention as a consequence of lesioning the area of the basal forebrain that supplies cortical cholinergic innervation. In one well-studied paradigm, such lesions interfere with the ability of rats to detect and respond to a briefly presented target stimulus that can appear in any of several locations (five-choice reaction time task) (Muir et al 1994, Robbins et al 1989). Those lesions decrease the accuracy of performance, an effect that can be overcome by increasing the target duration, which suggests that the impairment is attentional in nature. Impairments in the ability of aged rats to detect targets in the five-choice reaction time task that resemble the effects of basal forebrain lesions in young rats have recently been reported (Jones et al 1995). Another paradigm, a spatial cueing task originally designed for studies of attention in human beings, has also shown sensitivity to lesions of the basal forebrain in monkeys (Voytko et al 1994). Although the lesion methods used in this line of research with laboratory animals, until recently, have been relatively nonselective, removing both cholinergic and noncholinergic neurons in the basal fore-

brain, studies using a selective immunotoxin for cholinergic neurons have successfully produced deficits in attention when the cortical cholinergic projections are removed (Chiba et al 1995a,b).

The role of the basal forebrain cholinergic system in attention may extend to the component of this system that provides innervation of the hippocampal formation. As noted in an earlier section of this review, removal of those neurons with the selective immunotoxin fails to reproduce deficits in spatial tasks that are readily observed in aged rats (Baxter et al 1995a). In the chapter by Everitt & Robbins, the effects of less selective lesions are cited as evidence that the septohippocampal cholinergic system plays a role in memory. However, whether any substantial deficit in memory is observed with selective removal of these cholinergic neurons has yet to be demonstrated. In contrast, young rats with selective immunotoxic lesions of the cholinergic neurons that project to the hippocampal formation do have a marked impairment in a task in which attentional processing is modified in intact young rats (Baxter et al 1995b). The task involves repeated exposure to a cue that is subsequently used as a conditioned stimulus in associative learning. Preexposure to the cue usually retards subsequent learning, a phenomenon referred to as latent inhibition. Although more than one psychological explanation of latent inhibition has been offered, a number of explanations converge on the concept that decrements in attention to, or processing of, the preexposed cue serve as a basis for latent inhibition. In this context it is notable that either selective damage to the hippocampus (Han et al 1995) or selective removal of the cholinergic projection to the hippocampus impairs latent inhibition in rats (Baxter et al 1995b). The concept that a latent inhibition deficit might exist in aged rats because of diminished function of the cholinergic projection to the hippocampus has not yet been tested directly. It is interesting to note, however, that a recent study showed that information encoding of neurons in hippocampus in young rats will become unresponsive to cues that are not reliable features of a spatial environment, an effect not seen to the same extent in aged rats (Tanila et al 1996). Thus, apparently the selection of information that is subject to processing and encoding by hippocampal neurons is altered during aging in a manner that might be predicted from the effects of removing cholinergic neurons in young rats.

CONCLUSION

As information accumulates about the alterations that occur during aging in the brain, it becomes increasingly clear that a number of different types of changes can be identified in different neural systems. Moreover, the severity of age-related changes in particular brain systems often coincides with the extent of decline in cognitive functions associated with those systems. Certain evidence

has also indicated that heterogeneity in the effects of aging may exist in different cognitive domains and neurobiological systems. All these lines of evidence suggest that aging is not a global process, a conclusion that can be applied to the information derived from animal models as well as human studies.

In those cases where comparisons can be made across studies of human beings and laboratory animals, an important insight into the neurobiology of aging is emerging. Research using advanced stereological methods indicates that neuron loss is not characteristic of cortical systems, including the hippocampus, but that neurodegeneration does affect distinct populations of subcortical neurons that provide cortical innervation. Apart from such structural features of the brain, other aspects of functional integrity are also affected during aging. Although many of the detailed analyses available from studies of animal models have yet to be extended to studies of human brains, neuroimaging research provides support for the concept that processing within the existing circuits of the brain can be compromised during aging.

The comparison of functional analyses across species, including neuroimaging research with human beings, highlights the need to advance new models for understanding the neurobiological basis of cognitive alterations that occur late in life. Aging differs from many conditions involving brain damage, which neuropsychological studies were originally intended to address. Animal models for those conditions frequently entail the virtual destruction of a brain system to test hypotheses about the underlying substrate for cognitive functions of interest, e.g. the medial temporal lobe and declarative memory. During normal aging, in contrast, substantial structural integrity is preserved over the entire life span, and neurons that do exhibit appreciable neurodegeneration, such as those in the basal forebrain cholinergic system, are by no means eliminated. For this reason, lesion models may have limited utility for capturing the performance of neural systems in the aged brain, in which considerable remodeling occurs and a variety of functional alterations within the existing systems can be detected (Gallagher et al 1994).

Finally, the theme of individual differences in aging is well supported by studies of laboratory animals, including behavioral models developed for their sensitivity to memory functions subserved by the medial temporal lobe. Moreover, individual differences in behavioral capacities within these models often correlate with the severity of neurobiological alterations in the relevant brain systems. These lines of research give credence to the concept that age-associated memory decline in human beings can reflect a normal aging process, as distinct from a preclinical condition that heralds dementia. An understanding of the basis for individual differences in the effects of aging is likely to be advanced by further studies on the neurobiology of aging using animal models

as an important adjunct to the study of human beings. In this endeavor, it will be particularly important to determine the factors that distinguish those aged individuals that maintain preserved function from those that experience decline, an undertaking that will benefit from the bridges that can be formed between human beings and the study of aging in well-developed animal models.

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IN THE MATTER OF
US Serial No: 09/147,490
entitled"Neuroactive Peptide"

EXHIBIT 3

This is Exhibit 3 referred to in Clause 9 of the Statutory Declaration Siew Yeen Chai dated 13th Day of January 2004.

Before me: _____

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BRAIN RESEARCH REVIEWS

Review article

Acetylcholine: a neurotransmitter for learning and memory?

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Abstract

The cholinergic hypothesis claims that the decline in cognitive functions in dementia is predominantly related to a decrease in cholinergic neurotransmission. This hypothesis has led to great interest in the putative involvement of the cholinergic neurotransmission in learning and memory processes. This review aims to assess the data of studies in which the role of acetylcholine (ACh) in cognitive functions was investigated. For this purpose, studies from three different fields of research, namely: (1) behavioral pharmacology (effects of drugs on behavior); (2) behavioral neuroscience (effects of brain lesions on behavior); and (3) dementia, are discussed separately. The experimental tools that have been used in pharmacological studies may appear to be inadequate to enable conclusions to be drawn about the involvement of ACh in learning and memory processes. Especially, the use of scopolamine as a pharmacological tool is criticized. In the field of behavioral neuroscience a highly specific cholinergic toxin has been developed. It appears that the greater and more specific the cholinergic damage, the fewer effects can be observed at the behavioral level. The correlation between the decrease in cholinergic markers and the cognitive decline in dementia may not be as clearcut as has been assumed. The involvement of other neurotransmitter systems in cognitive functions is briefly discussed. Taking into account the results of the different fields of research, the notion that ACh plays a pivotal role in learning and memory processes seems to be overstated. Even when the role of other neurotransmitter systems in learning and memory is taken into consideration, it is unlikely that ACh has a specific role in these processes. On basis of the available data, ACh seems to be more specifically involved in attentional processes than in learning and memory processes.

Keywords: Acetylcholine; Alzheimer's disease; Cholinergic; Cognition; Dopamine; Glutamate; Learning; Memory; Muscarine; Nicotin; Serotonin

Contents

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1. Evidence for a cholinergic involvement in learning and memory

The notion that cognitive functions are highly dependent on central cholinergic neurotransmission goes back to the early sixties (for a detailed historical overview, see [7,58,111]). Although other neurotransmitters were known to be involved in learning and memory performance, the functions of the cholinergic system in learning and memory were of predominant interest to learning and memory research [52]. The first experimental studies that evaluated the role of the cholinergic synapse in the storage and retrieval of new information were performed by Deutsch [36]. Since then, different fields of research have provided an empirical foundation for the cholinergic hypothesis of learning and memory: (1) behavioral pharmacology (effects of drugs on cognition); (2) behavioral neuroscience (effects of brain lesions on cognition); and (3) aging and dementia research. The last field of research is probably the keystone of the cholinergic hypothesis of learning and memory. Moreover, the importance of the finding that the cognitive decline in aging and dementia is related to a decrease in cholinergic function can be deduced from the fact that the effects of cholinergic antagonists and lesions of cholinergic nuclei are often related to cognitive deficits similar to those observed in aging and dementia (e.g., [30,40,67,73]).

The experimental findings from the different fields of research which support the cholinergic hypothesis are summarized below (a more detailed conceptual/empirical overview has been given by Bartus and colleagues [7]).

1.1. Behavioral pharmacology

There are numerous pharmacological studies which have evaluated the effects of cholinergic antagonists and cholinomimetics on learning and memory performance [52,82,120]. The cholinergic muscarinic antagonist scopolamine is the drug most widely used to induce amnesia in experimental subjects. The first experimental studies with this drug were performed by Drachman and Leavitt [40], who reported that scopolamine induced amnesia in young healthy subjects. This loss of cognitive abilities appeared to be comparable to that observed in old untreated subjects. In addition, acetylcholinesterase inhibitors, which enhance the availability of acetylcholine (ACh) in the synaptic cleft, were able to reverse the scopolamine induced deficit, indicating that the cognitive deficit is cholinergic in nature. The peripheral effects of scopolamine can be controlled for by including a control group treated with methylscopolamine, a substance which does not readily cross the blood–brain barrier but which has peripheral effects similar to those of scopolamine. The characteristics of the scopolamine model suggest that the cognitive deficits that can be observed after scopolamine treatment are directly related to a decrease in central cholinergic functions. Other cholinergic drugs, which have distinct effects on cholinergic neurotransmission (e.g., the choline uptake inhibitor hemicholinium, the specific muscarinic type 1 receptor antagonist pirenzepine and the nicotinic antagonist mecamylamine), also have a negative effect on learning and memory performance.

1.2. Behavioral neuroscience

Many studies in which specific brain areas are lesioned have been performed to relate the cholinergic deafferentation of different brain structures with a decline in cognitive performance [52,120]. A cholinergic dysfunction can be induced by either lesion of cholinergic nuclei or transection of major cholinergic pathways. These methods have shown that specific disturbances in learning and memory performance are observed after a cholinergic lesion. Most consistent and dramatic are the effects on learning and memory observed after cholinergic lesions which affect the hippocampus [53,127]. This can be achieved by transection of the fimbria-fornix, or lesion of the vertical limb of the diagonal band of Broca, or of the medial septum [80]. During the last decade much attention was given to a model in which the nucleus basalis magnocellularis was lesioned in experimental animals. Lesions of this nucleus decrease cortical choline acetyltransferase (ChAT) activity and the learning and memory performance declines [120]. Further support for a cholinergic involvement in this model came from studies which showed that scopolamine exacerbates the performance of nucleus basalis lesioned rats whereas ACh esterase inhibitors improve it [120]. Cholinergic-rich transplants have been found to reverse the nucleus basalis lesion induced performance deficit [42], providing additional support for the cholinergic nature of the

cognitive performance deficit in experimental animals with a cholinergic lesion.

1.3. Aging and dementia

Aging is characterized by a deterioration of memory functions, a deterioration which will affect almost everyone during the last decades of life. A more severe deterioration of cognitive functions is observed in patients suffering from Alzheimer's disease (AD). Although it remains controversial whether cholinergic markers (ChAT, ACh levels, number of cholinergic cells) decline during 'normal aging' [32], a clear consistent decline in cholinergic markers has been found in AD [6]. In fact, studies have shown that there is a relation between the decrease in cognitive functions and markers of the cholinergic system in senile dementia ([101]; for review see [27]). Because there was no correlation between the cognitive decline in dementia and other neurotransmitter systems [97], the conclusion reached was that the decline in the cholinergic system underlies the cognitive deficits of dementia.

1.4. Aims of this review

The cholinergic hypothesis derives its strong status from the fact that it is supported by results from these different fields of research. Thus, ACh could be considered to be a neurotransmitter which is highly involved in learning and memory processes. However, there are studies in all the three fields of research which do not support this hypothesis. Moreover, the validity of experimental data from pharmacological and lesion studies, which were interpreted in terms of cholinergic mechanisms, has been seriously questioned in recent publications [43,46]. The aim of this review is to critically re-evaluate the involvement of the cholinergic system in learning and memory processes. Each of the three different fields of research described above will be considered separately. Emphasis is given to those studies that do not support the well-established cholinergic hypothesis and the impact these studies could have on the viability of the hypothesis. Also, the possible role of other neurotransmitter systems and the interactions between neurotransmitter systems in learning and memory performance will be discussed. Finally, some conceptual reflections on the interpretation of the behavioral data obtained with learning and memory tasks are discussed.

2. Behavioral effects of cholinergic drugs

Many studies have shown that cholinergic antagonists mediate the behavioral impairment observed in different learning tasks. As mentioned above, in most studies scopolamine was used to induce a performance deficit. Most effects obtained in these studies (performance deficits and the reversal of scopolamine-induced deficits by various drugs) were interpreted in terms of a cholinergic effect on learning and memory. However, it first has to be established whether the performance deficits in these tasks are truly due to the effects of cholinergic drugs on learning and memory processes. Possible side effects of cholinergic drugs that could have a negative effect on the performance of a task that assesses cognition should be excluded. Further, many stages can be distinguished in the processing of information and subsequent memory formation: e.g., perception, sensory processing, associative processing and memory consolidation. There are different processes which lead to the behavioral output, e.g., retrieval of experience, constellation of motor programs, coordination of motor systems and muscle contractions. Each of these processes could be affected by drugs. Since these processes eventually affect behavior, conclusions about the effects of drugs on learning and memory processes can only be drawn if the effects on other processes can be excluded, or their contribution can be distinguished experimentally.

A number of studies have evaluated the effects of scopolamine on sensory, attentional and motor functions. These studies have been extensively reviewed by Hagan and Morris [52]. In a discrimination task in which rats had to respond to an intermittent light stimulus, scopolamine decreased the stimulus sensitivity at doses of only 0.06 mg/kg (i.p.) [130]. Spontaneous locomotor activity is increased at a scopolamine doses of 1 mg/kg (i.p.) [104]. Nictitating membrane conditioning is also retarded after scopolamine treatment in rabbits, which may suggest a disruption of learning processes [54]. However, in additional experiments it was shown that higher tone intensities were needed to elicit a response in scopolamine-treated animals than in saline-treated animals. It was suggested that scopolamine blocks the excitatory properties of tone stimuli at a dose of 0.4 mg/kg (i.v.). Thus, the impaired learning performance in this task could be explained by a scopolamine-induced impairment of sensory processing. Even low doses of scopolamine (0.005–0.375 mg/kg, i.p.) affected a sensitive discriminability measure in a delayed visual conditional discrimination task [63]. That visual discrimination performance is very sensitive to cholinergic drugs has also been shown in another study [2] in which scopolamine (0.078–0.5 mg/kg, i.p.) and methyl-scopolamine (0.3–0.5 mg/kg, i.p.) increased response latencies and the number of missed trials in a visual discrimination task. Surprisingly, methyl-scopolamine also affected the accuracy of the performance whereas scopolamine did not. This could suggest a peripheral effect of cholinergic drugs on performance in this test. Related to this, it has been argued that methyl-scopolamine can cross the blood–brain barrier and, dependent on the behavioral parameter, affect behavior by central cholinergic neurotransmission [83].

In young monkeys scopolamine (0.03 mg/kg, i.m.) induces a delay-dependent deficit in a delayed matching-to-sample task [110]. This impairment showed a similar profile when the delay was lengthened, irrelevant stimuli

were presented during the delay interval, or when the performance was compared with that of old rats. In contrast to the performance-enhancing effects of physostigmine in young scopolamine-treated animals in the standard task, physostigmine did not improve the performance of old monkeys. Also, physostigmine did not improve the performance of young monkeys in the task in which prolonged delays were presented or in the distraction task. It was therefore suggested that the short-term memory performance in this task might not be mediated by the cholinergic system. An alternative explanation for these data was that scopolamine could have disrupted attentional functions.

Experimental evidence for a decrease of attentional abilities after scopolamine in animals has also been extended to humans. Several studies have provided experimental evidence that scopolamine affects the allocation of attentional processes rather than impairs memory per se in humans [41,131]. Considering the data mentioned above, peripherally administered scopolamine may not primarily and/or selectively affect learning performance and memory processes but rather affect sensory/attentional processes [52,63,119].

It remains to be shown whether the effects of scopolamine on stimulus processing and attentional processes are generally applicable to the same class of cholinergic antagonists, or whether this effect is typical for scopolamine. Increased behavioral activity can be observed after systemic injection of atropine and scopolamine at doses of 4 and 8 and 1 and 2 mg/kg, respectively [104], suggesting that muscarinic antagonists affect behavioral activity. The effects of other cholinergic drugs on sensory processing, attention or behavioral activity, have, to my knowledge, only been evaluated to a limited extent (e.g., [3]). Some experimental evidence shows that scopolamine and mecamylamine have distinctive effects on performance in learning and memory tasks [28,85]. This suggests that muscarinic and nicotinic receptors have dissociable behavioral functions. The role of nicotinic receptors in cognitive processes will be discussed in greater detail later.

A further point that should be mentioned with respect to the specificity of scopolamine is that scopolamine-induced amnesia is attenuated/antagonized by different compounds which affect other neurotransmitter systems (e.g., D-amphetamine, piribedil, fluoxetine) or receptor channels (e.g, strychnine, picrotoxin). For instance, ACTH [47], nootropic drugs (e.g., piracetam, aniracetam) and plant extracts [56] have been found to reverse the performance deficits induced by scopolamine (see [120]). These 'anti-amnesic' effects of different classes of drugs questions the cholinergic specificity of the behavioral effects in two respects. First, it could be argued that the non-cholinergic actions of drugs enhance memory performance by increasing attention/arousal. Secondly, the effects of scopolamine might not be specific for the cholinergic system [58].

Another problem with the interpretation of the above mentioned studies is that they examined the effects of cholinergic drugs after systemic injection. However, the cholinergic system is distributed throughout the whole brain. Different pathways can be distinguished which project to the cortex, bulbus olfactorius, hippocampus and/or amygdala [80] and these pathways may have qualitatively different psychological functions [55]. Thus, it could be argued that the effects of cholinergic drugs are undifferentiated because the entire cholinergic neurotransmission is affected after systemic injection [46,64]. This problem can be circumvented by injecting a drug in a specific region of the brain. Several studies have shown that local infusion of cholinergic drugs into the hippocampus impairs performance in learning and memory tasks [12,15,18]. However, a detailed analysis of the performance (e.g., signal detection theory measures, effects on motor functions) of the rats was not performed and the results of these studies may therefore be not conclusive with regard to the effects of the (hippocampal) cholinergic system in learning and memory processes.

It has been shown that bilateral intrahippocampal injections of scopolamine (30 μg in a volume of 0.5 μl) and pirenzepine (35 μg, volume 0.5 μl) (M_1 receptor antagonist) have different effects on the performance in a T-maze alternation task [79]. Scopolamine injections impaired the accuracy of performance but also increased the response latency. Pirenzepine also impaired the accuracy of performance but had no effect on the response latency. Additional support for a more specific involvement of the M_1 receptor antagonist comes from a study in which the effects of unilateral intraventricular injections of pirenzepine (1–93.2 μg, volume 5 μl) were evaluated in a spatial learning task in the Morris water tank [51]. Thus, a more receptor subtype-specific muscarinic antagonist may have more specific effects on learning and memory than the non-selective muscarinic receptor antagonist scopolamine. That drugs with different specificities for subpopulations of cholinergic receptors have different effects on delayed responding performance in a matching to position paradigm was nicely demonstrated in a recent study by Andrews and colleagues [3]. They reported that unilateral intraventricular administration of muscarinic receptor blockers (pirenzepine, AFDX 116 (M_2), UH-AH 37 (M_1/M_3); all drugs tested at doses of 3.2–32 μg, volume 5 μl) were more effective in disrupting the accuracy of performance than were nicotinic receptor antagonists (mecamylamine, hexamethonium), although it must be mentioned that the nicotinic drugs were administered i.p. In addition, they found that hemicholinium-3 (0.2–5 μg, volume 5 μl) and to a lesser extent pirenzepine, affected the accuracy of performance without having significant effects on motor performance. These findings demonstrate that subpopulations of cholinergic receptors have different effects on behavioral parameters.

Bilateral intra-hippocampal injection of scopolamine

(4–35 μg, volume 1 μl) have been found to cause a delay-dependent performance deficit in a delayed matching to position task, whereas scopolamine injections into the prefrontal cortex cause a delay-independent deficit [45]. The delay-dependent deficit suggests that the hippocampal cholinergic system is specifically involved in short-term memory processes. The prefrontal cholinergic system appeared to affect performance in a non-specific manner and therefore it was suggested that this system does not have a specific role in short-term memory. However, in another study in which scopolamine (5–15 μg, volume 0.5 μl) was injected bilaterally in the prefrontal cortex, a delay-dependent effect was found in a similar task, suggesting a role for the prefrontal cholinergic system in short-term memory [19]. At present no ready explanation can be given for the discrepancy between these two studies, although some differences existed with respect to the task characteristics (correction procedure in study of Broersen and colleagues [19] but not in the study of Dunnett et al. [45]). Clearly, more studies are needed to resolve this apparent controversy.

Bilateral intra-amygdaloid injections of scopolamine (8–72 μg, volume 0.5 μl) have been found to affect working memory to a greater extent than reference memory in a double Y-maze [59]. The effects of bilateral intrahippocampal mecamylamine injections (8–72 μg, volume 2 μl) on cognitive performance have been evaluated in a three-panel runway. These data provide evidence for a role of hippocampal nicotinic receptors in working but not reference memory [94]. This same group reported comparable data in a similar study with pirenzepine and methoctramine (both drugs tested at doses of 0.1–1 μg, volume 2 μl, [95]). That is, cholinergic drugs affected the measure working memory, whereas reference memory appeared not to be affected. However, these three studies did not examine possible non-specific effects of the treatment. Therefore, that these treatments might have affected, for example, sensory processes and/or motivational aspects, cannot be excluded. Another feature of the findings of Ohno and collegues [95] is that blockade of M_1 and M_2 receptors had similar effects. This is somewhat unexpected since blockade of M_2 receptors is assumed to enhance the release of ACh and appears to improve performance in the spatial Morris task [39,106].

One study evaluated the effects of unilateral intraventricular injections of scopolamine, infused continuously (0.004–40 μg/kg/h), on the behavior of monkeys [22]. The performance of two monkeys was examined in a continuous performance task in which the animals were required to locate a briefly presented stimulus on a TV monitor during a session of 150 trials. At a dose of 12.5 $\mu g \cdot kg^{-1} \cdot h^{-1}$, scopolamine decreased the number of responses. This decrease was not associated with a decrease in accuracy. In addition, the effect became more apparent when performance was examined during trials with short stimulus presentations and towards the end of the session. Although only two monkeys were tested, these data suggest that the effects of scopolamine treatment could be related to an impairment of attentional functions.

The data from studies in which drugs were injected locally indicate that the psychological functions of the cholinergic system may differ in different brain areas and that the functions of the cholinergic system can be dissociated on the basis of the different receptor subtypes involved. Thus, studies in which cholinergic drugs are injected into defined structures of the brain may provide more pharmacological evidence for a possible role of the cholinergic system in cognitive processes. Although data from pharmacological experiments in which drugs are injected intracerebrally may have a greater value than data from studies in which drugs are injected systemically, the drawbacks of intracerebral injections should be recognized (e.g., diffusion of drugs to other areas, [81]; electrophysiological abnormalities [109]; lesion at the site of the injection; within group variation in coordinates of injection site).

That the extent of diffusion of drugs into different brain areas can be a problem was recently demonstrated by Jolas and colleagues [62]. They showed that 8-OH-DPAT diffused about 0.4 cm from the injection site. The diffusion of drugs depends on their biophysical properties, injection volume, rate of injection and the brain structure in which the drugs are injected. Nevertheless, local application of selective cholinergic drugs should be preferred above systemic injections when the functional role of the cholinergic systems is to be assessed. Moreover, the apparent distinctive role of cholinergic subtypes [3,33] and the distinctive psychological functions of different cholinergic systems [46,50] suggest that the conceptual interpretation of studies in which non-specific cholinergic drugs are injected systemically is limited.

Most data from psychopharmacological studies with scopolamine are likely to be explained in terms of non-mnemonic effects. Various studies have shown that scopolamine has distinct effects on sensory processing [54,63]. Moreover, the use of scopolamine as a pharmacological model for the cognitive deficits in dementia can be questioned since the cognitive deficits after scopolamine treatment are very dissimilar to those observed in dementia [9,118]. It is therefore disappointing that scopolamine (often at too high doses) has been used in many studies. Whether other cholinergic drugs with a high specificity for cholinergic receptor subtype(s) would specifically affect learning and memory processes awaits further well-designed studies. These studies should also examine dose–response relationships since these provide more insight into the effects of the drug being investigated.

A more convincing manner to provide experimental evidence for a role of the cholinergic system in learning and memory processes would be to show that procholinergic compounds improve performance. There is, in fact, ample evidence that, in animals as well as in humans,

nicotine improves performance in different cognitive tasks (for review see [70]).

Although enhanced performance after nicotine treatment could be interpreted in terms of learning and memory enhancement, an explanation in terms of attentional improvement cannot be excluded. Support for such an assumption comes from a study in which the effects of nicotine (0.1 and 0.4 mg/kg s.c.) and mecamylamine (2.5 and 5 mg/kg s.c.) were investigated in adult and aged rats in a delayed responding task in a T-maze [133]. In this study nicotine improved working memory performance (at all delays) in old rats but not in adult rats. Since the old rats had a poor working memory performance at the shortest delay, the poor performance could be interpreted in terms of an impairment in attention and/or stimulus discrimination. Consequently, it was argued that the improved performance of the old rats after nicotine treatment could be best explained in terms of the processes that impair performance, i.e., attentional processes. Additional support for the notion that nicotine may enhance memory performance in AD by means of improved attentional functions comes from the studies of Sahakian and colleagues [112,113].

There is abundant evidence that nicotine also interacts with the serotonergic, dopaminergic, glutamatergic and GABAergic neurotransmitter systems [35,70]. As will be discussed later, these neurotransmitter systems have also been found to affect behavioral performance in learning and memory tasks. Bearing this in mind, the contribution of cholinergic neurotransmission to the enhanced performance observed after nicotine treatment cannot be dissociated from the effects of nicotine on the other neurotransmitter systems. Interpreting the effects of nicotine in patients with dementia who have deficits in multiple neurotransmitter systems in terms of a cholinergic effect may not be justified. Data about experimentally 'pure' cholinergic deficits would be more informative about the possible cholinergic effects of nicotine on learning and memory processes than those observed with AD patients. Various experimental studies have shown that nicotine attenuates cognitive deficits in different models of cholinergic deficits [34,57,125]. However, as has mentioned above, there is no pharmacological model in which cholinergic performance deficits affect only learning and memory processes. In other words, there are no cholinergic drugs available that induce 'pure' learning and memory deficits. Whether there is a 'pure' cholinergic brain lesion model in which cholinergic cognition enhancers can be tested, will be discussed in the next section.

3. Behavioral effects of cholinergic lesions

The observation that dementia may be related to a decline in cholinergic neurotransmission gave rise to studies of the involvement of the cholinergic system in mnemonic functions in experimental animals. This was achieved by lesioning cholinergic nuclei. Since cortical and hippocampal areas receive their major cholinergic input from the cholinergic nuclei of basal forebrain [80], these areas were selected for lesion studies. Lesions of the nucleus basalis in young animals resulted in marked performance impairments in different learning and memory tasks in different species [27,120]. Moreover, the observation that cortical ACh markers were also lower after forebrain lesions underscored the notion that cholinergic neurotransmission has a crucial role in lesion-induced performance deficits (for an overview, see [43]). Although basal forebrain lesions appeared to be a simple animal model of the cognitive deficits of dementia, recent studies do not further support this model. After ibotenic acid lesions of the nucleus basalis, cortical ChAT activity was maximally depleted by about 45%. Basal forebrain lesions with other neurotoxins, such as quisqualic acid and AMPA, did lead to a higher depletion (70%) of cortical choline acteyltransferase activity, but were far less able to impair the performance in learning and memory tasks (see [43]). Thus, the contribution of the cholinergic system in the behavioral deficits seen after nucleus basalis lesions appears to have been overestimated in early studies. Moreover, the data point to a divergence in the decline in cholinergic markers and behavioral changes.

More recently, studies have been performed in which distinct forebrain lesions were made with a highly selective cholinergic neurotoxin, 192 IgG Saporin. This toxin binds specifically to the low-affinity p75 NGF receptor, which is predominantly localized on cholinergic cells [134]. The first study that reported the effects of this selective neurotoxin on behavior showed that spatial orientation learning was impaired in lesioned animals [92]. The toxin was injected intracerebroventricularly and resulted in a dramatic decrease in ChAT activity in the hippocampus (85–90%) and cortex (65%) but not in the striatum or mesencephalon. More specific lesions of the basal forebrain were made in additional studies with this toxin. When the nucleus basalis magnocellularis of rats was lesioned with an optimal amount of the toxin, a high selectivity to cholinergic cells was confirmed [132]. However, no learning and memory deficits were observed in these rats. It should be noted that the cortical ChAT activity in these animals was decreased by only about 30%.

In another study 192 IgG saporin was injected into the medial septum, nucleus basalis and lateral ventricle [11]. The impairments in learning and memory performance were most dramatic in the ventricle-injected group and confirmed the results of the study of Nilsson and colleagues [92]. Septal lesions had virtually no effect on performance in a spatial orientation task whereas nucleus basalis lesioned animals showed a clear deficit in this task. ChAT activity was not evaluated in this study, but in both groups an almost complete loss of AChE staining was

observed in the hippocampus and cortex. The learning impairment of the ventricular-injected animals was discussed in terms of an effect of the toxin on cerebellar Purkinje cells. These cells also express the p75 NGF receptor [102] and, indeed, a loss of Purkinje cells was observed in the ventricular-injected animals. Considering the role of the cerebellum in conditional learning [68], the behavioral deficits of the ventricular-injected group are probably better explained in terms of an effect of a cerebellar lesion.

The results of another study failed to confirm the behavioral deficits seen after nucleus basalis lesions with 192 IgG saporin [126]. Rats with a lesion of the nucleus basalis showed virtually no deficits in learning and memory in different types of tasks (i.e., spatial orientation, delayed conditional discrimination). In fact, a modest impairment in inhibitory avoidance performance was observed in these rats. In this lesion group, cortical ChAT activity was reduced by 75–90%. In agreement with the study of Berger-Sweeney and colleagues [11], no behavioral deficits were observed after septal lesions or lesions of the diagonal band of Broca (reduction in hippocampal ChAT activity: 65–72%). The study of Torres and colleagues [126] also demonstrated the cholinergic specificity of the lesion. Thus, it appears that the more specific the cholinergic lesion, the less dramatic the effects on learning and memory performance. Extrapolation of these data would suggest that a complete and selective cholinergic lesion would have no effect on learning and memory.

Although no effects on learning and memory are observed after selective cholinergic lesions, a cholinergic involvement in attentional functions has been suggested. Ibotenic acid lesions of the basal forebrain, with magnetic resonance imaging being used to determine the position of the basal forebrain cholinergic nuclei in individual monkeys, did not appear to affect the monkeys' cognitive performance [129]. Although no mnemonic deficit could be detected, the lesioned monkeys showed a deficit in attentional focusing. Cortical ChAT was found to be decreased by 40–60% (hippocampal and amygdaloid ChAT activity was not evaluated). It was concluded that functions of the basal cholinergic forebrain are involved in attentional processes rather than in mnemonic processes. Studies with rats support the notion that the basal forebrain cholinergic nuclei could have an important role in attentional functions. Using a multiple-choice serial reaction time task, Muir and colleagues found that attentional functions to be mediated by the cholinergic basal forebrain [87,89]. The absence of dramatic learning and memory impairments in 192 IgG saporin-lesioned rats made different authors assume that the cholinergic system is involved in attentional functions [126,132]. It should be mentioned that the first studies with the selective cholinergic neurotoxin did not show substantial learning and memory impairments when distinct nuclei were lesioned. A possible cholinergic involvement in learning and memory might become evident when multiple cholinergic nuclei are lesioned. Although this has to be tested in further experiments, the results of Voytko and colleagues [129] do not support this idea.

4. A cholinergic deficit in dementia

Although the relation between an age-related decline in cognitive functions and the cholinergic system is not clear, considerable experimental evidence exists for a relation between the decline in cholinergic functions and dementia [6,97,101]. The correlation between cognition and the cholinergic system in dementia re-instated the idea that the cholinergic system is involved in learning and memory and boosted research into the involvement of the cholinergic system in learning and memory. In addition, there was a parallel with Parkinson's disease in which a neurotransmitter deficiency (dopamine) is related with motor dysfunctions. Since treatment with a dopamine precursor is quite successful in Parkinson patients, there was great hope of a cholinergic treatment for dementia. This approach offered great advantages since clear targets could be defined for the development of different cholinergic drugs. Although the correlation between the cholinergic deficiency and the cognitive decline in dementia led to the simple conclusion that a cholinergic deficit underlies the cognitive deficit, several data argue against such an inference (see [1]).

First, cholinergic pharmacotherapies have been remarkably unsuccessful thus far. The different approaches that have been pursued aimed to increase ACh levels in the clefts of cholinergic synapses or to mimic its effects at distinct ACh receptors. It could be argued that currently available drugs are not specific enough and that more specific drugs (i.e., drugs that selectively bind to specific cholinergic receptors, or ACh esterase inhibitors with less adverse effects) will be more successful in alleviating the cognitive impairment in dementias. Sarter and colleagues [84,116,117] provided another explanation for the lack of effect of this strategy to enhance cholinergic neurotransmission. They argued that a direct enhancement of the cholinergic system may disengage the still-intact cholinergic neurotransmission because non-activated ACh efflux is stimulated. This tonic stimulation of the cholinergic neurotransmission is not expected to have beneficial effects on behavior. In contrast, modulation of the cholinergic system by trans-synaptic mechanisms (i.e., affecting cholinergic neurotransmission by modulating GABAergic neurotransmission) may be more efficient in alleviating the cognitive deficits since this amplifies the physiological cholinergic signal. Such an approach could be achieved by using a benzodiazepine partial inverse agonist [116].

A second argument that contradicts the presumed relation between ACh and cognitive performance comes from

studies which have shown that there is an overlap in ChAT activity in healthy old people and in patients with AD [17,98,100]. Related to this, a third criticism against a role of cortical cholinergic system in cognition comes from observations from patients with inherited olivopontocerbellar atrophy. It appears that in these patients cortical, but not hippocampal, ChAT activity is decreased to a similar extent as observed in AD [65]. However, the dominantly inherited olivopontocerebellar atrophy is not associated with the disabling dementia characteristic of AD. This has led authors to assume that a cholinergic deficit in the hippocampus, but not the cortex, is responsible for the observed cognitive decline in AD [99]. Although this may be the case, it remains intriguing that cortical ChAT activity correlates with cognitive functions in dementia [101], but that low ChAT activity does not affect cognitive functions in the olivopontocerbellar atrophy [65]. The possible implications for this observation will be given later.

A final criticism (see [1]) is that the cholinergic hypothesis does not take into account the correlation between neurofibrillary tangles (a hallmark of AD) and dementia [91]. Thus, although the conclusion was simple and straightforward, the cholinergic hypothesis does not account for all the observations in dementia.

Although several arguments can be given against a cholinergic involvement in the cognitive deficits in dementia, it cannot be denied that dementia is associated with a dramatic deterioration in cholinergic markers. The logical question is why the cholinergic system is especially vulnerable to the neurodegenerative process in dementia. Three hypothetical explanations of the observed cholinergic deficit in AD are given below.

Wurtman offered an explanation for the increased vulnerability of cholinergic cells in neurodegenerative diseases [135]. He argued that cholinergic neurons have a unique metabolic capability as choline is used for two purposes. First, the acetylated form of choline is used as the neurotransmitter of these neurons. In addition, as in all other cells, choline is transformed to phosphatidylcholine, which is needed to maintain cell membranes. In AD phospholipid metabolites are increased and choline levels are decreased, which suggests that choline metabolism is disturbed. Under these low choline conditions, cholinergic cells appear to use membrane-bound choline for neurotransmission. This process has been called 'autocannibalism' [16]. It was further argued that the disturbed membrane composition could eventually expose the transmembrane domain of the amyloid precursor protein to proteolytic enzymes, which in turn could augment the conversion of the amyloid precursor protein into the abnormal amyloid form. This hypothesis thus links the cholinergic deficit and the abnormal amyloid deposit.

A decline in central energy metabolism might also explain the cholinergic deficit in dementia. Beal and colleagues [8] forwarded the hypothesis that a disturbed energy metabolism is the underlying cause of neurodegenerative diseases. Moreover, they found experimental evidence that oxidative damage to mitochondrial DNA could underlie mitochondrial dysfunctions (i.e., decrease in energy metabolism) in AD [77]. As yet, it remains unclear whether the production of free radicals precedes the energy decrease or the energy disturbance precedes the increased formation of free radicals. Either way, this cascade leads to a decreased availability of energy resources. That a disturbed energy metabolism could particularly affect cholinergic cells may be related to the fact that choline is used for two purposes (see above). In addition, it has been argued that an inhibition of energy metabolism could affect the metabolism of the amyloid precursor protein and thereby contribute to the characteristic amyloidosis seen in AD [48].

Others have proposed that disturbances in the metabolism of glucose underlie the cholinergic deficit in AD [78]. Although this may have similarities with the above-mentioned hypothesis of mitochondrial damage, which also leads to a decreased energy production, the decrease in ACh and the formation of amyloid are explained by different mechanisms in this model. A lower turnover of glucose leads to lower levels of acetylcoenzyme A, which is necessary for the acetylation of choline to ACh. Consequently, lower levels of choline are available under low energy conditions. Low glucose levels may also explain the formation of amyloid plaques. If too little ATP is produced, as it is in AD, the amyloid protein cannot be incorporated into the membrane and deposits of amyloid are formed. Since the membranes are no longer able to 'rebuild' under these conditions, cells progressively shrink and eventually die.

The three hypotheses are speculative and more studies are needed to test their validity. But, if a decrease in central energy metabolism underlies the neurodegeneration of dementia, a cholinergic deficit is likely to be one of the early signs of dementia. This is consistent with the findings of many studies in which a decrease in cholinergic markers in AD has been observed. However, the observed decline in cholinergic markers does not necessarily explain the decline in cognitive functions in dementia. A correlation between two parameters does not necessary indicate that there is a causal relation between the two parameters. In this respect it should be mentioned that there are other parameters (e.g., neurofibrillary degeneration, loss of pyramidal cortical neurones) that correlate with the clinical symptoms of dementia [91]. If there is a decrease in energy metabolism, the functioning of all neurons will be affected and the cognitive disturbances may be related to a general poor functioning of the nervous system. Collerton [27] proposed five possible models to explain the cholinergic deficit and the cognitive decline in dementia. In one model, which was one of two possible hypotheses at that time, it was assumed that neurodegeneration underlies the cognitive decline in AD as well as the cholinergic deficit but that these two phenomena were independent of each

other. This is consistent with the hypothesis mentioned above.

As mentioned before, various neurotransmitter systems, catecholaminergic [97] as well as excitatory amino acid [1,98], are affected in AD. Considering the role of these neurotransmitter systems in learning and memory (see next section), it is not surprising that cognitive functions deteriorate in dementia. However, no clear correlations between neurotransmitter levels and cognitive performance have been observed for neurotransmitter systems other than the cholinergic system [98]. Nevertheless, the correlation between cholinergic markers and cognitive functions in dementia have been questioned by different authors (see [1,98]). Although the two energy hypotheses remain speculative, they do offer an explanation for the correlation between cholinergic markers and dementia and may explain the decrease in multiple neurotransmitter systems.

Several studies have evaluated the hypothesis that a reduced availability of energy sources affects brain chemistry and behavior. This was done by examining the effect of intraventricular injections of streptozotocin, which is assumed to compromise the metabolic state of neurons [93]. Although different neurotransmitter systems are affected after streptozotocin treatment [38], a clear correlation between hippocampal ChAT activity and spatial discrimination performance has been observed in rats [14,105]. These first studies support a possible relation between energy disturbances, decline in cholinergic markers and cognitive dysfunctions. However, more studies are needed in which the consequences of centrally administered streptozotocin or other metabolic inhibitors are evaluated at the behavioral as well as the biochemical level to evaluate the viability of the 'energy hypothesis'.

To summarize, the decrease in cholinergic markers in dementia could be regarded as an epiphenomenon of this neurodegenerative disease. This conclusion is supported by the observation that the cholinergic markers are decreased in the dominantly inherited olivopontocerebellar atrophy to a similar extent as in AD whereas olivopontocerbellar atrophy is not associated with a cognitive deficit and that cortical ChAT is correlated with the cognitive deficit in dementia. Thus it would appear that the decrease in cholinergic neurotransmission does not have a crucial role in the cognitive dysfunctions of dementia.

5. Involvement of other neurotransmitters in learning and memory performance

The aim of this section is not to review the role of the different neurotransmitter systems in learning and memory processes. Instead, the aim is to give an impression of the extent to which other neurotransmitter systems are involved in the performance of learning and memory tasks. This will help us to compare these effects with those obtained in studies with cholinergic agents. Three different classes of neurotransmitters, serotonin, dopamine and excitatory amino acids are considered, as these have received the most attention in the literature thus far. In addition, the interaction between the cholinergic system and other neuromodulatory systems will be discussed.

5.1. Serotonin

Serotonergic neurotransmission has been suggested to have a crucial role in different psychiatric disorders [20]. In addition, the role of serotonin in learning and memory has also received much interest although these data appear to be rather inconsistent (see [72]). However, experimental data suggest that stimulation of serotonergic neurotransmission impairs behavioral performance, whereas inhibition of the system enhances performance. Promising data have been obtained with 5-HT_3 antagonists [4], which have been found to improve the performance of rodents and primates in various cognitive tests [5]. It may therefore not be surprising that different compounds have been developed for the treatment of AD (e.g., ICS 205930, Ondansteron and Zacopride; see [115]). Different studies have also provided a possible neurochemical mechanism of action. It appears that 5-HT_3 receptors modulate cortical ACh release and may possibly act via an additional 5-HT receptor subtype (see [4]). The effects on ACh release in the cortex are assumed to strengthen the apparent cognition enhancing effects of the 5-HT_3 antagonists.

Because there is a high density of 5-HT_{1A} receptors in the entorhinal cortex [103], a structure that is highly involved in learning and memory functions, it has been assumed that this receptor subtype could be a putative target for cognition-enhancing drugs. Various studies investigated the role of the 5-HT_{1A} receptor subtype in learning and memory, most of which failed to provide evidence for an improvement of learning and memory after treatment with 5-HT_{1A} agonists [23,24,61]. In fact, no effect or an impairment of learning performance was observed. Only one study has shown that a selective partial agonist of the 5-HT_{1A} receptor (ipsapirone) improves performance in a conditional delayed discrimination task ([26], but see [61]). Whether a 5-HT_{1A} antagonist can improve performance in learning and memory tasks remains to be demonstrated.

5.2. Dopamine

Dopamine has been a subject of intensive research, mainly because of involvement in psychiatric disorders, in the (mis)use of psychostimulants and in Parkinson's disease. The role of dopamine in learning and memory has received relatively little attention, although different studies have provided experimental evidence that it modulates learning and memory performance in different types of tasks. Especially, the mesocortical dopamine system is thought to have a crucial role in cognitive processes

because this neurotransmitter has an important role in the functions of the prefrontal cortex [66,69]. Various studies have indeed observed learning and memory performance deficits in animals with dopaminergic lesions (see [69]). A distinction in the functions of dopaminergic neurotransmission in learning processes has been proposed on basis of the receptor subtypes D_1 and D_2, [10,90]. It has been suggested that D_1 receptors are important in the maintenance of reward-related learning, whereas D_2 receptors appear to be functionally related to the type of reinforcer involved.

The role of dopamine in learning and memory performance, as assessed in mazes and delayed response tasks, is not clear. For example, post training administration of quinpirole (D_2 agonist) and D-amphetamine (catecholaminergic agonist) has been found to improve performance in various types of tasks (see [96]). Others have reported an impairment of short-term memory in a delayed response task after administration of D-amphetamine [21]. This deficit appeared to be related to the D_2 and D_3 receptor subtypes. It could be argued that dopamine enhances learning processes but interferes with memory processes. A more conceptual interpretation of the role of the (mesotelencephalic) dopaminergic system has been proposed by Dunnett and Robbins [44], who suggested that this system is functionally related to the convergence of information about reward (the prefrontal cortex and amygdala) and visual processing and attention (neocortical input to striatum). A very elaborate review by le Moal and Simon [69] provided a (holistic) view on the functions of the dopaminergic system. They suggested a role of the dopaminergic network in terms of the regulation of internal homeostasis, whereby dopamine is assumed to function as a sensor.

5.3. Excitatory amino acids

Glutamate, the most abundant endogenous excitatory amino acid in the brain, has received increasing attention since its proposed involvement in neurological and psychiatric disorders (see [74]). A clear connection between excitatory amino acids and learning and memory-related processes is provided by the involvement of NMDA and AMPA receptors in the induction of long-term potentiation. Long-term potentiation has been suggested to be the physiological correlate of memory formation [60]. Although data are available that both long-term potentiation and memory can be disrupted by blockade of the NMDA receptor [86], the enhancement of the glutamatergic signal may have adverse effects on behavior because high levels of glutamate are neurotoxic [25]. Therefore, there may be only a small window for memory-enhancing effects of excitatory amino acid receptor agonists. Nevertheless, cognition enhancing effects have been observed with a newly developed drug (i.e., 1-(1,3-benzodioxol-5-ylcarbonyl)piperidine), which was assumed to facilitate the functioning of AMPA receptors, in different learning and memory models [122]. Finally, there is a glutamatergic deficit in AD (see [1]). However, it remains to be determined whether this is related to high glutamate levels, which induce neurotoxicity, or whether this is related to a decrease in glutamatergic function.

5.4. Interaction between different neuromodulators

The separate discussion of the different neurotranmsmitter systems may give the impression that the different neurotransmitters act separately and have distinct and even crucial, roles in learning and memory processes. But the improved performance cannot necessarily be interpreted in terms of improved memory storage. For example, one should be aware of the putative antidepressant and anxiolytic effects of serotonergic drugs [20], especially in avoidance tasks. The improved performance observed after administration of dopaminergic drugs could be related to attentional processes, effects on motor functions and reward mechanisms [35,44]. NMDA receptors have been found to have pronounced effects on sensory information processing [29], a mechanism by which performance could be improved or impaired. Thus, as with the cognition-enhancing effects of nicotine, the improved performance in cognitive tasks could be mediated by processes other than learning and memory.

Considering the complexity of the neuronal networks in the brain, it would be naive to assume that one neurotransmitter regulates such a complex mechanism as learning and memory. It is perhaps wiser to assume an interactive framework in which the different neurotransmitter systems are involved in learning and memory. Decker and McGaugh [35] presented an integrative model in which the cholinergic neurotransmission has a central role. The cholinergic system appears to be interconnected with norepinephrine, dopamine, serotonin, GABA, opioid peptides, galanin, substance P and angiotensin II. The authors suggested that the interaction between the cholinergic system and the other neuromodulators could be essential for the formation of memory. Indeed, this view is gaining greater acceptance and the involvement of different neurotransmitter systems in cognitive performance has been recognized in different recent studies ([49,71,107,114,124]; for review see [35,123]). Further investigation of the interaction between the different neurotransmitter systems in learning and memory performance will be a challenging manner to gain more knowledge of the role of these neurotransmitter systems in the cognitive control of behavior.

6. Assessment of learning and memory

I have tried to avoid interpreting the performance-enhancing/impairing effects of drugs and lesions on behav-

ior in terms of 'enhancement/impairment of learning and/or memory processes'. Mostly, the effects are referred to as an enhancement/impairment of 'learning and memory performance' and 'cognitive performance'. This was done because of the definitions of learning and memory. According to Vanderwolf and Cain [128], there is confusion about the definition of learning and memory because one line of research conceives learning and memory as a psychological process, whereas another line conceives learning and memory as a change in synaptic neural connectivity. Consequently, the interpretation of the concepts of learning and memory is also rather confusing according to Vanderwolf and Cain [128]. Their conceptual reorientation is pessimistic with respect to the behavioral neurobiology of learning and memory and Vanderwolf and Cain state that there is not a test that specifically assesses memory. It is true, the many definitions of learning (e.g., [37]) and the various memory systems (e.g., [121]) that have been postulated have not contributed to an unambiguous interpretation of the results of different studies, let alone, permitted parallels between studies with different species to be drawn.

It was not the aim of this review to discuss the problems that exist concerning the definition of learning and memory, although the reader should be aware that these problems exist. Knowing that learning and memory cannot be given a unique definition, how do we interpret behavioral parameters in a task when these parameters change on repeated testing? Too often, without knowing what could have been the cause for a behavioral change, any observed behavioral change has been interpreted in terms of learning and memory. Task-specific characteristics that could have influenced the performance of the 'control' and 'experimental' group to a different extent, are not generally considered. For example, age-related deficits in spatial discrimination performance have often been interpreted in terms of deficits in spatial information processing. However, a more detailed evaluation of the spatial learning performance of young and old rats and taking into account the physiological changes that occur with aging, shows that age-related performance deficits are not necessarily related to changes in the ability to process spatial information (e.g., [13]). Related to this, conclusions about the cognition-enhancing effects of a drug should not rest on the results of one experiment. The effects of a drug should be evaluated in different types of tasks that tap different aspects of behavior to exclude alternative explanations for the enhanced performance in a learning/memory task [75]. This will necessitate elaborate studies in which the behavioral effects of experimental manipulations are evaluated on several behavioral domains.

I consider that behavioral changes should be interpreted in terms of the parameter under investigation rather than boldly in terms of learning and memory processes. Thus, when treatment with a drug leads to longer latencies to enter the dark compartment in inhibitory/passive avoidance tests, it should not be stated that the drug has memory enhancing effects. Instead, the effect of the drug should be interpreted in terms of longer latencies to enter the dark compartment. Any other interpretation is liable to anthropomorphism. Treatments that lead to longer step-through latencies in inhibitory/passive avoidance tests do not necessarily imply an improved learning and memory performance. An anecdotal example of this is the assumed memory-enhancing effect of vasopressin, a peptide that was found to 'improve' performance in avoidance tasks [31]. Several alternative explanations have been offered for the improved performance after vasopressin treatment (see [30]). Further, the parameter under investigation may not reflect the process that is assumed to be measured, i.e., the test parameter may not be valid. In addition, it should be remembered that a selected behavioral parameter may reflect a constitution of various behavioral dimensions. Thus, a learning parameter such as 'swim path' in the Morris water escape task may not only reflect the ability to learn the position of the hidden platform but also reflect exploratory aspects of behavior.

A more concise interpretation of behavioral data (i.e., related to the parameter under investigation) could possibly avoid confusion about the interpretation of the effects of treatments. The behavioral effects of drugs in so-called learning and memory tasks do not necessarily imply that the treatment affects learning and memory processes. In other words, defining a task as a learning task does not imply that there is a one-to-one relation between the behavioral change and a learning process. Other possible explanations for the behavioral change should be ruled out before the behavioral change can be interpreted in terms of learning and memory. As this may seem not be possible, one should be careful with interpreting behavioral data. As long as there are no widely accepted definitions of learning and memory, this terminology should preferably not be used.

7. Concluding remarks

The aim of this review was to critically assess the data used to investigate the role of cholinergic neurotransmission. Since it was my aim to demonstrate that the assumed role of ACh in learning and memory processes probably has been overestimated, much of the 'cholinergic literature' has that supports the involvement of ACh in cognitive processes not been cited. This does not imply that the present review did not take this pro-cholinergic literature into account. Much of the cited literature is of recent date and therefore reflects the latest progress in this field. In addition, the conclusions of the various studies have been related to the established cholinergic hypothesis. Thus, the cholinergic literature has not been neglected. I sought experimental support for my conclusions in three different fields of research, namely psychopharmacology, behav-

ioral neuroscience and dementia. A brief summary of the findings in the three different research fields is given below.

In 1988, Hagan and Morris [52] came to the following conclusion: "Much of the psychopharmacological data, particularly the rodent experiments, are confusing, contradictory and/or open to several interpretations. Nevertheless, the literature reveals a number of well-executed studies which, having controlled for confounding variables, produced data most parsimoniously explained in terms of a cholinergic involvement in memory mechanisms." (p. 301). Since then, many studies have been carried out to investigate a cholinergic involvement in cognition. New cholinergic drugs with a high specificity for cholinergic receptor subtypes and newly developed learning and memory tasks have enabled researchers to investigate the role of receptor subtypes in learning and memory performance in greater detail. In addition, some tasks (e.g., the delayed matching to position task) allowed evaluation of the possible side effects of the pharmacological treatment simultaneously. Nevertheless, it is my opinion that these pharmacological studies do not allow us to make a more definite conclusion about a cholinergic involvement in memory than that made by Hagan and Morris. Thus, the developments in this field of research have not brought us closer to untangling the role of the cholinergic system in learning and memory. Although a role for ACh in learning and memory processes is at present still a matter of discussion, data from pharmacological studies suggest that ACh has a role in attentional processes [30,88,89]. The interpretation of pharmacological studies is often limited by the pharmacological tools used in studies. Moreover, the distinctive role of different cholinergic systems, the side effects of cholinergic drugs and the functional dissociation of cholinergic receptor subtypes have only recently been appreciated.

Substantial progress has been made in the search for a highly selective cholinergic neurotoxin [134]. This experimental tool may be very useful in evaluating the role of the different cholinergic systems in cognitive processes. A clear distinction between the selectivity of a cholinergic lesion and the occurrence of cognitive deficits has been found, namely the increased selectivity of cholinergic neurotoxins (associated with increased cholinergic deafferentiation) resulted in fewer dramatic cognitive deficits in experimental animals [43,108,126]. Thus, this research provides experimental evidence that cholinergic neurotransmission may not be critically involved in learning and memory processes, although it may have a role in attentional/arousal processes [87,126,129].

The correlation between cortical ChAT and cognitive performance in dementia appears not to be so clearcut as originally assumed [101]. Especially, the lack of cognitive deficits in patients with inherited olivopontocerbellar atrophy gave rise to skepticism as to the role of cortical ChAT in cognitive processes [65]. Alternative hypotheses for the neurodegeneration seen in AD were discussed. It was assumed that a disturbance in central energy metabolism could underlie the neurodegeneration, especially that of cholinergic neurons and decline in cognitive functions in AD. Although speculative, according to this hypothesis the decrease in cholinergic markers in dementia could be regarded as an epiphenomenon. Support for a role of the cholinergic system in cognitive functions in AD may come from the pharmacological studies that showed an improvement in attentional functions after treatment with cholinergic drugs (i.e., tacrine and nicotine [112]).

On the basis of data from the different research fields, the following conclusions can be made. The experimental data that have been obtained in lesion studies do not support the notion that ACh plays a pivotal role in learning and memory processes. Also, in AD, a neurodegenerative disease in which many neurotransmitter systems are affected, it is not evident that the decline in cholinergic markers predominantly explains the reduced cognitive abilities. Bearing in mind the criticism of the psychopharmacological studies, it may be too early to draw conclusions about the cholinergic involvement in cognitive functions. Thus, two different research lines do not support the notion that ACh has a significant role in learning and memory. It is therefore concluded that ACh has only a limited role in the formation of memory and that its role in cognitive functions has been overestimated to date.

The focus on ACh as a neurotransmitter primarily involved in learning and memory does not take into account the possible involvement of other neurotransmitter systems in cognitive functions. The role of other neurotransmitter systems in behavioral changes in learning and memory tasks was briefly discussed. The literature seems to suggest that, for example, excitatory amino acids are more important in the formation of memory than ACh. Several authors have postulated that ACh may have a pivotal role in attentional processes [87,112,129] and the data from the various research fields that have been discussed here support this behavioral function of ACh.

If it is assumed that cholinergic neurotransmission is primarily involved in attentional processes, two points should be borne in mind. The first is related to the difference in outcome of pharmacological and lesion studies. The second is related to the observation that selective cholinergic lesions have only modest effects in learning and memory tasks.

(i) There are apparent differences in the results obtained from psychopharmacological and lesion studies. Lesion models have been developed in which specific cholinergic systems can be lesioned. This may be comparable with the level that can be achieved with pharmacological tools when drugs are injected locally and block cholinergic neurotransmission. Just as lesions and pharmacological modulation are two completely different tools, the results from these two fields of research are also qualitatively different. Locally applied drugs have clearcut effects on performance in different tasks of learning and memory

(e.g., [19,45,79,95]), whereas specific cholinergic lesions seem to have only modest effects on behavioral performance [126,129]. This dissociation is difficult to explain. Moreover, cholinergic lesions do not distinguish between different receptor subtypes whereas cholinergic drugs have different affinities for receptor subtypes. Thus, it could be expected that the effects of lesions have a greater impact than pharmacological manipulation of cholinergic receptor subtypes. Resolving this gap may be a way to obtain a better understanding of the cholinergic system in behavioral functions.

(ii) Selective cholinergic lesions in the basal forebrain system appear to have only a modest effect on behavior in learning and memory tasks [11,126,129,132]. Since it can be assumed that attentional processes are involved in the primary stages of information processing, it is intriguing that no dramatic effects in various learning and memory tasks have been observed [126,129]. If attentional processes are affected, a performance deficit would be expected especially in delayed response tasks. However, it appears that this is not the case after selective cholinergic lesions. This highlights the need to develop tests that measure attentional processes (e.g., [76,84,87]). Since there are different types of attentional processes (e.g., divided attention, focused attention, sustained attention), various tasks should be developed that can distinguished different processes. This would enable us to investigate the function of cholinergic neurotransmission in these processes. The minimal effects that have been observed in learning and memory tasks after selective cholinergic lesions may question the sensitivity of these tasks to measure performance deficits. It could be argued that the learning and memory tasks that are currently available do not measure only learning and memory processes [128]. It will be a challenge to develop more sophisticated test models to assess learning and memory processes in laboratory animals.

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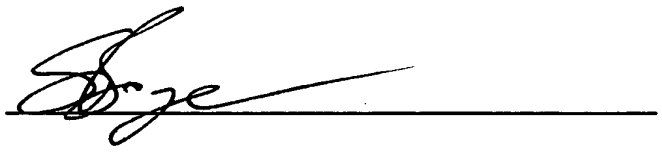
IN THE MATTER OF
US Serial No: 09/147,490
entitled"Neuroactive Peptide"

EXHIBIT 4

This is Exhibit 4 referred to in Clause 10 of the Statutory Declaration Siew Yeen Chai dated 13th Day of January 2004.

Before me: 

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Alzheimer's disease and Down's syndrome: roles of APP, trophic factors and ACh

Ole Isacson, Hyemyung Seo, Ling Lin, David Albeck and Ann-Charlotte Granholm

Recent therapeutic investigations of Alzheimer's disease (AD) have been guided by two seemingly opposed hypotheses: the amyloid cascade theory, which favors the amyloid plaques as the cause of AD; and the cholinergic theory, which favors cholinergic neuron loss as the cause. New investigations indicate that the synthesis and processing of the amyloid precursor protein (APP) is linked to the trophic actions of nerve growth factor. A pathological cascade in both AD- and Down's syndrome-related memory loss could be triggered by alterations in APP processing or ACh-mediated neuronal function, or both, which in turn trigger the overexpression of amyloid β, synaptic malfunction and trophic factor loss in target regions. This eventually leads to synaptic and dendritic loss with age.

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In Alzheimer's disease (AD)- and Down's syndrome (DS)-related dementia, the progressive nature of neurodegeneration suggests an age-dependent process that ultimately leads to degeneration of synaptic afferent systems, dendritic and neuronal damage, and the formation of abnormal protein aggregates throughout the brain. By the fourth decade of life, individuals with DS display many of the same neuropathological features (i.e. neuritic plaques, neurofibrillary tangles and degeneration of basal forebrain cholinergic neurons) as do individuals with AD, and many of these individuals develop dementia early in life [1–5]. It has been suggested that the neurodegenerative processes in AD and DS are closely related and at least partially comparable [4].

Recent findings indicate that cortical and hippocampal cholinergic synaptic systems [6,7] and trophic factors [8] can either reduce or accelerate pathogenesis and progression of AD and DS pathology by effects on amyloid precursor protein (APP) levels, metabolism and processing. The pathology of AD is determined diagnostically by several standardized clinical and pathological criteria [9–12]. Cortical plaques, neuronal tangles and degeneration of afferents to areas such as the hippocampus and neocortex are neuropathologically linked in a multifactorial progression. The exact physiological sequence of events leading to these pathologies is unknown.

The early discovery of ACh deficiency [13] singled out the loss of this neurotransmitter as one reason for the cognitive dysfunction in AD. Recent reports have detected only slight biochemical enzyme loss in the cholinergic system in mild cognitive impairment, which is thought to be the precursor to AD [14–17], although these studies have not determined whether the function of the cholinergic system is intact. Many elegant studies support the cholinergic hypothesis [18,19], showing that a dysfunctional cholinergic system is sufficient to produce memory deficits in animal models that are analogous to Alzheimer's dementia. Nevertheless, to date, little information exists about the dynamic processes that underlie the cognitive loss and neuropathology.

The cholinergic system (and potentially other afferent systems such as the noradrenergic and serotonergic systems), APP and trophic factors are intimately linked in normal function and possibly also in pathogenesis. This article provides a synthesis of these pathophysiological interactions and new interpretations of available data and recent findings.

Transgenic, mutant and animal models

Recently, a transgenic mouse has been described that expresses a neutralizing monoclonal antibody against nerve growth factor (NGF) [8]. In these mice, brain pathology exhibits remarkable similarities to the pathology seen in progressive AD, including amyloid plaques, hyperphosphorylated tau, neurofibrillary tangles in cortical and hippocampal regions, and marked cholinergic neuron degeneration. The anti-NGF transgenic mice show more pathology resembling that found in individuals with AD than transgenic mice that express mutant APP [8]. In fact, studies using the transgenic mice with human mutations of presenilin or APP, or both, have failed to demonstrate significant alterations in the cholinergic cell body region, even though in the APP–presenilin double mutant (APPK670N, M671L and PS1M146L [20]) there is a decrease in the density of cholinergic synapses in the frontal cortex [21].

Another study (using the human APP overexpression mutant, V717F [22]) also failed to detect significant degeneration of the cholinergic neurons, even at 18 and 26 months of age, despite significant levels of amyloid plaques in the brain. These findings from transgenic mice indicate that there are factors other than faulty amyloid processing involved in the neuropathology of AD.

Consistent with the findings using anti-NGF transgenic mice [8] described above, a toxic antibody (anti-NGF) treatment paradigm in rats leads to deterioration of cholinergic CNS basal forebrain neurons and synapses, and produces a shift towards APP elevations and decreased levels of choline acetyl transferase (ChAT), which are associated with behavioral deficits in spatial orientation and memory

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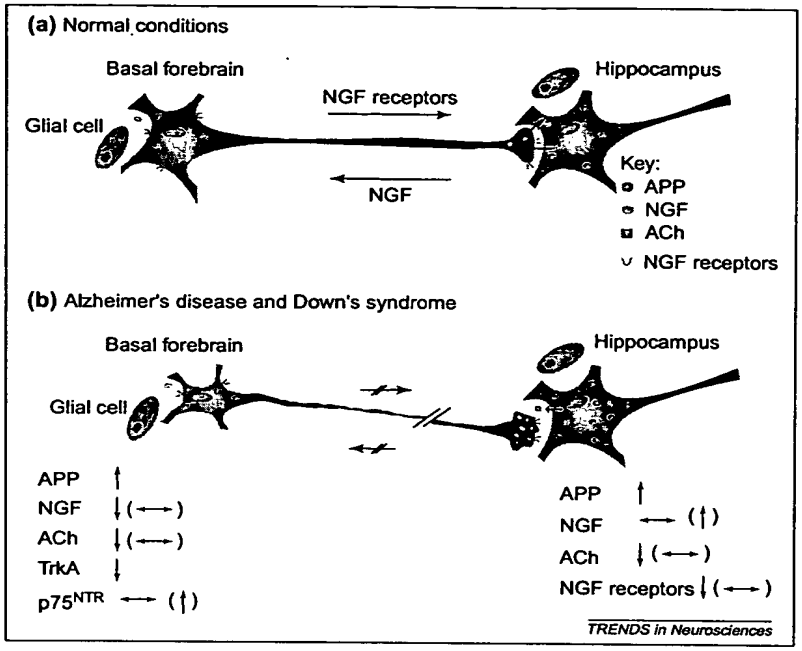

Fig. 1. Cholinergic basal forebrain neurons (a) in normal physiological conditions and (b) as postulated in individuals with AD and DS. In cholinergic neurons of the basal forebrain in individuals with AD and DS, ChAT immunoreactivity, cell size and number, and NGF and TrkA levels are decreased. In the hippocampus of individuals with AD and DS, ChAT levels and ACh-mediated signaling are reduced but NGF levels are increased or unchanged. In the hippocampus and basal forebrain of individuals with AD and DS, APP expression and Aβ aggregate levels are increased, whereas secreted (trophic) sAPPs are decreased. There are also degenerating terminals in the hippocampus. These altered levels indicate that NGF retrograde transport or NGF binding to trkA receptors, or both, are reduced in the individuals with AD, which results in inappropriate trophic support of the cholinergic system during degenerative disease [78]. Abbreviations: Aβ, amyloid β; ACh, acetylcholine; AD, Alzheimer's disease; APP, amyloid precursor protein; ChAT, choline acetyl transferase; DS, Down's syndrome; NGF, nerve growth factor; sAPP, soluble APP; TrkA, tyrosine receptor kinase A.

tasks [6,23]. In addition, studies have been performed on mice with a segmental trisomy of chromosome 16, Ts65Dn [24,25]. The triplicated gene segment includes the gene for APP, and other genes that are present in individuals with DS [26]. These mice

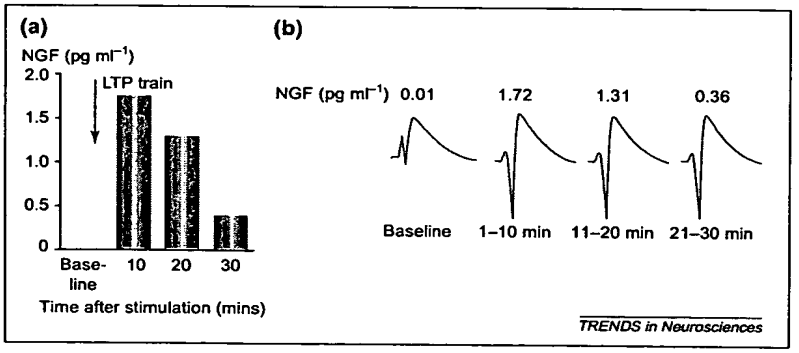

Fig. 2. Long-term potentiation (LTP)-induced nerve growth factor (NGF) release in a hippocampal slice from a young rat. (a) LTP was induced by rapid-train stimulation with an electrode, and NGF was measured by a specific NGF enzyme-linked immunosorbent assay kit in the superfusate after protein purification at different times after stimulation (10, 20 and 30 min). (b) The field potentials show the LTP induction and the NGF concentration in pg ml^{-1}. These data suggest a dynamic interaction between hippocampal activity and NGF release that could regulate synaptic terminal growth and function [67,68]. These regulatory release patterns were later also confirmed for another member of the neurotrophin family, brain-derived neurotrophic factor (BDNF) [69].

overexpress full-length APP and amyloid β, and undergo significant deterioration of the cholinergic system and basal forebrain NGF levels as young adults [24]. Ts65Dn mice exhibit reductions in basal forebrain cholinergic neuron size and number, and regressive changes in the hippocampal terminal cholinergic axonal fields, which are thought to be associated with impaired retrograde transport of NGF from hippocampus to the basal forebrain [27,28].

All of these recent animal models re-emphasize the possibility that pathological processes operating in age-related cognitive impairment, idiopathic AD and DS might be related to physiological changes in several systems, including synaptic complexes associated with cholinergic nerve terminals. Such synapses depend on the trophic action of NGF for their function [29–32]. As cholinergic degeneration in individuals with AD is closely correlated with the degree of memory impairment [13], this transmitter system and related trophic factors might also be associated with amyloid plaque formation and related trophic factors, neuronal tangles and cell degeneration (Fig. 1).

APP-related systems interact with both trophic factors and cholinergic receptors

A high percentage of basal forebrain cholinergic neurons degenerate in AD [5]. The cholinergic neurons are highly dependent upon NGF for their function [33–36], and use high-affinity tyrosine receptor kinase A (trkA) and low-affinity (p75) receptors for signaling. The trkA receptors are synthesized in cholinergic neurons and are necessary for binding and retrograde transport of the growth factor from the terminals to the cell body region, while the low-affinity receptors appear to be important for sequestering growth factor molecules to the presynaptic membrane [37–40]. However, NGF is synthesized by neurons in the target regions and is released in an activity-dependent manner [35,41,42] (Figs 1–3).

In individuals with AD, there is typically a marked loss of high-affinity trkA receptors in both cholinergic target neurons and basal forebrain neurons, which correlates with loss of cholinergic neurons [43–45]. Even though there have been some variable results regarding NGF protein levels in different brain regions of individuals with AD, most recent studies agree that there are unchanged or increased NGF levels in the hippocampus and cerebral cortex, while the levels in the basal forebrain are decreased compared with age-matched controls [46–53]. This, together with cholinergic neuron loss and dysfunction, suggests that NGF is not adequately transported retrogradely to the basal forebrain or that binding to the high-affinity receptors or release of NGF from hippocampal interneurons, or both, is deficient in the brain of individuals with AD and in animal models of aging (Figs 1,3) [49,54].

Infusion of NGF can prevent degeneration of axotomized cholinergic CNS neurons [55]. A biological basis for an interaction between APP and NGF has

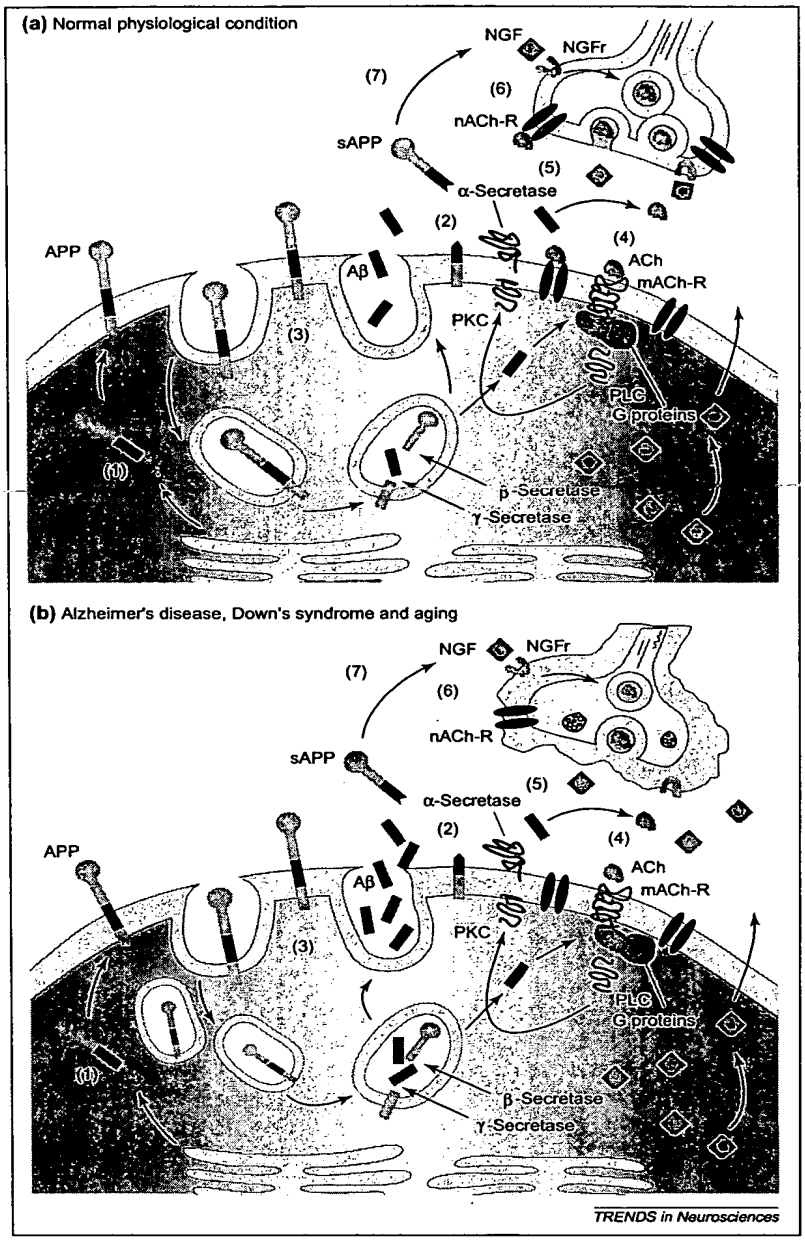

Fig. 3. Different pathways for amyloid precursor protein (APP) processing and nicotinic-receptor- and muscarinic-receptor-mediated regulation of APP metabolism (nACh-R and mACh-R, respectively). (a) (1) Newly synthesized APP is transported from the Golgi through vesicles to the cell surface (2) where it can be cleaved within the amyloid β (Aβ) domain by α-secretase. Mature cell-surface APP can also be reinternalized (3) into late endosomes and lysosomes, where it can be processed by β-secretase and γ-secretase to yield Aβ, which can be rapidly secreted into the extracellular fluid. (4) Acetylcholine (ACh) is released because of the arrival of an action potential. It binds to α7 nicotinic receptors (α7 nACh-R) presynaptically, and α7, M_1 and M_2 receptors postsynaptically. The α7 nACh-R binding results in presynaptic Ca^{2+} influx, which leads to increased ACh-mediated tone. Released ACh from presynaptic terminals also binds to G-protein-coupled muscarinic receptors and subsequent activation of phospholipase C (PLC), which hydrolyzes phosphoinositolipids to inositol-3-phosphate and diacylglycerol. Diacylglycerol activates protein kinase C (PKC), which directly or indirectly enhances α-secretase-mediated cleavage of APP. (5) Aβ can interfere with cholinergic neuron function at both presynaptic and postsynaptic signaling sites (4) and (5), and increased ACh-mediated signaling leads to decreased APP levels and increased release of soluble APP (sAPP). (6) Acetylcholine release increases NGF secretion from the postsynaptic membrane, which binds to presynaptic NGF receptors (NGFr), resulting in retrograde transport of NGF to the cell body of cholinergic neurons. (7) The signaling of NGF to NGF receptors is stimulated by sAPP. (b) In Alzheimer's disease and Down's syndrome, pathological conditions and aging, there is damage to terminals and (1) increased APP expression, (2) increased Aβ secretion by amyloidogenic processing, (3) internalization of mature cell-surface APP, (4) reduced ACh neurotransmission, (5) increased Aβ interference of ACh signaling, and (6) reduced transport of NGF–NGFr complexes to the ACh cell body.

been suggested by cell culture studies that demonstrate a dose-dependent relationship between NGF and APP messenger induction [56–58]. After exposure to NGF, primary cortical neuronal cultures showed increased levels of membrane phospholipids that might promote APP expression and secretion of the soluble form of APP (sAPP). The large membrane-spanning precursor molecule (full-length APP) can be processed into several different biologically active compounds, such as the secreted form, sAPP, which has been shown to have neurotrophic activities, and the longer aggregating forms, of which amyloid β_{1-42} is the most toxic [53,58,59] (Figs 1,3). Cell culture work also indicates that sAPP potentiates the effects of NGF on differentiation of catecholaminergic cell lines [60]. Soluble APP can also augment the effects of NGF, and, conversely, the expression and release of APP is temporally enhanced by NGF *in vivo* [58]. A trophic relationship between sAPP and NGF has thus been established.

The cholinergic system has been shown to have a regulatory effect on both APP and NGF-related processes. Some types of cholinergic neuron lesions can increase hippocampal NGF levels in the normal adult rodent brain [61–63]. Other findings connect muscarinic-receptor agonists and other ACh-related agents with NGF and APP. For example, recent studies have demonstrated that amyloid β_{1-42} binds to α7 nicotinic ACh receptors with high affinity [64]. In the same study, it was also shown that the blocking effect of amyloid β_{1-42} on the presynaptic nicotinic ACh receptors gave rise to decreased Ca^{2+} influx, and thus inactivation of the presynaptic membrane (hence, a decreased ACh-mediated tone was produced by amyloid β_{1-42} administration).

On the basis of these and other recent *in vivo* findings using ACh M_1-receptor agonists [8,9,65], it is postulated that close relationships exist between the function of trophic factors, APP and neurotransmitters such as ACh in regulating the health of neurons (Figs 1,3). A pivotal pathological cascade in both AD- and DS-related memory loss might be triggered by alterations in APP processing or cholinergic neuronal dysfunction, or both, which triggers overexpression of amyloid β, synaptic malfunction and trophic factor loss in target regions (eventually leading to synaptic and dendritic loss in all regions involved). By this and other genetic mechanisms, abnormal levels of neurotrophic

sAPP and pathogenic levels of solubilized amyloid β might eventually cause progressive (and regressive) degeneration of cholinergic nerve terminal function in target regions (hippocampus and cerebral cortex), and thus decreased ACh-mediated tone, which leads to decreased NGF release and uptake, cholinergic neuronal cell body atrophy, and metabolic downregulation (Figs 1,3).

NGF, LTP and the cholinergic hippocampal synapse

Given that long-term potentiation (LTP) has been used as a model for synaptic plasticity and learning in the hippocampal formation, it is relevant to determine whether NGF is associated with LTP [64]. Indeed, evidence shows a significant increase in basal NGF release after LTP induction in hippocampal slice cultures (Fig. 2) [67,68]. These regulatory release patterns were later confirmed for another member of the neurotrophin family, brain-derived neurotrophic factor [69]. That hippocampal target levels of NGF are increased in aging and sometimes in AD [46], seems to contradict the degeneration and atrophy seen in cholinergic neurons, but is explained by disease and age-dependent mechanisms of reduced NGF uptake by cholinergic nerve terminals [49].

LTP induces an acute and specific release of NGF in the hippocampus, which is consistent with reports that ACh-receptor agonists have this effect [42]. However, chronic over-stimulation by ACh-receptor agonists *in vivo* in young adult mice leads to a compensatory reduction in hippocampal NGF levels, probably as a feedback signal to lower cholinergic nerve terminal function and growth (Fig. 1) [70]. Interestingly, local application of NGF to basal forebrain cholinergic neurons can give rise to a slow-onset (~20 min) but significant increase in basal firing rate, especially in cholinergic neurons in aged animals that have been shown to have decreased basal forebrain levels of NGF [68]. This increase in cholinergic neuron activity in response to locally administered NGF seen in aged animals might be an adaptive mechanism that recruits NGF to a system in need of trophic support. Thus, there is also a close relationship between NGF and this cholinergic pathway in rapid cellular events in the basal forebrain.

It is possible that this two-way relationship is altered by events that lead to AD and DS. Enhanced LTP and afferent synaptic strength via cholinergic, serotonergic and, possibly, noradrenergic systems in the hippocampal formation has been shown to enhance memory function. Conversely, impairment of these transmitter systems reduces LTP and memory function, at least in hippocampal-dependent tasks [24,71]. As previously discussed, this is modeled by transgenic expression of an antibody against NGF [8]. Such an antibody or toxin to the trkA receptor [6,23] selectively disrupts the ACh-mediated innervation of hippocampal neurons and the functional integrity of this system. Further evidence of a close relationship between ACh-mediated transmission and APP, is the increase in the levels of sAPP found after M_1-receptor activation. Soluble APP is considered to be a trophic substance in these systems [72–77]. Increased NGF levels seen in our studies after LTP (Fig. 2), combined with an increased release of sAPP under maximal activation of the cholinergic system would enhance NGF function. In any case, these intracellular changes and reduced metabolic functions are consistent with a dysfunction of the cholinergic system and trophic target zones in the hippocampal formation, and of other ACh-receptive regions of the cerebral cortex (Fig. 3) [65].

Comprehensive AD- and DS-like pathologies are thus produced by altered cholinergic and APP-related systems in trisomic mice that overexpress APP [27] or by *in vivo* NGF dysregulation via antibodies directly against NGF or its receptors [6,8,65]. Recent observations are consistent with the idea that there are homeostatic mechanisms regulating hippocampal NGF, APP and ACh-mediated activity. A dysregulation of one or more of these three factors would progressively lead to imbalance in neurotransmission, eventually leading to synaptic damage and neuronal cell loss relevant to memory function. Models that focus on complex interactions involved in dementias might be more realistic for achieving new drug, cellular and molecular therapies to influence both stage- and age-dependent neurodegenerative disease.

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A rationale for the structure of color space

R. Beau Lotto and Dale Purves

The colors perceived by humans in response to light stimuli are generally described in terms of four color categories (reds, greens, blues and yellows), the members of which are systematically arrayed around gray. This broadly accepted description of color sensation differs fundamentally from the light that induces it, which is neither 'circular' nor categorical. What, then, accounts for these discrepancies between the structure of color experience and the physical reality that underlies it? We suggest that these differences are based on two related requirements for successful color vision: (1) that spectra be ordered according to their physical similarities and differences; and (2) that this ordering be constrained by the four-color map problem.

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The goal of any visual system is presumably to distinguish physically different objects and the conditions under which they are witnessed, thus enabling successful visually guided behavior. All visual animals achieve this end by detecting differences in the quantity of the LIGHT (see Glossary) reflected by objects or otherwise returned to the eye, which are perceived in humans as LIGHTNESS and/or BRIGHTNESS. Many animals also distinguish objects according to differences in the quality of the light they reflect (i.e. the distributions of the spectral power in the stimulus), which are perceived in humans as different COLORS.

Although descriptions of the organization of human color sensations differ in detail [1–4], they share several key features. Thus, at any given level of light intensity, color experience can be portrayed as a plane in which movements around the perimeter correspond to changes in hue and movements along its radial axis correspond to changes in saturation (i.e. changes in the relative grayness of the color) (Fig. 1). In contrast to the continuously variable spectral distributions that generate sensations of color, all colors are experienced as belonging to one of four perceptual categories (reds, greens, blues and yellows), or combinations thereof. Thus, although the relationships between other visual sensations and the physical world that gives rise to them (e.g. sensations of shape, depth and motion) appear straightforward (i.e. the structure of physical space is roughly similar to the overall structure of the perceptual space it generates), color sensations are different: there is no obvious basis in the physical characteristics of light for either the circular ordering of colors in a plane, or their parcellation into four perceptual categories.

Why, then, is perceptual color space structured in this way, and does this structure have deeper implications for understanding the nature of vision generally? To the extent that contemporary theories of color vision have addressed these questions, the subjective structure of color experience is considered an inevitable consequence of TRICHROMACY and OPPONENCY ([5–9], but see Ref. [10]). Thus, most modern work has understandably focused on determining the cellular bases of these two physiological pillars of color sensation. As a result, the rationale for the structure of color experience is, in this view, secondary to the evolutionary value of trichromacy and opponency as such. Some of the advantages that have been suggested are: (1) optimally satisfying the constraints of information theory [11–13]; (2) promoting the perception of 'color constancy' [14–16]; and (3) helping our frugivore ancestors detect ripe fruit [17,18].

We take the opposite approach to understanding color experience. Rather than rationalizing the structure of color sensations in terms of trichromacy and opponency, we consider the structure of color space itself, asking whether color space (and thus the physiology that generates it) might represent the solution to the two fundamental problems in topology with which the evolution of color sensations must ultimately contend.

Distinguishing territories by spectral information

In examining the proposition that the structure of color experience, as such, should be a better guide to understanding color vision, a good place to start is to consider why color sensations have evolved in the first place. Many animals do not have a significant degree of color vision, and even those that do are for the most part more limited in color perception than are

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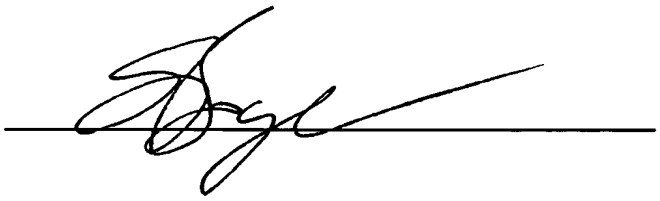
EXHIBIT 5

This is Exhibit 5 referred to in Clause 11 of the Statutory Declaration Siew Yeen Chai dated 13th Day of January 2004.

Before me: 

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Letters to the Editor

SELECTIVE LOSS OF CENTRAL CHOLINERGIC NEURONS IN ALZHEIMER'S DISEASE

SIR,—Alzheimer's disease is a progressive cerebral degeneration which continues without remission until death, usually in profound dementia. Morphologically, the disease is characterised by large numbers of senile plaques and neurofibrillary tangles in the brain, these tangles being especially abundant in the cerebral cortex. Neurochemical studies are still in their infancy, and we know nothing of the molecular basis of the disease.

We have studied the enzymes associated with the putative neurotransmitters acetylcholine, γ-aminobutyric acid, dopamine, noradrenaline and 5-hydroxytryptamine in twenty regions of brains obtained at necropsy from three patients with Alzheimer's disease and from ten individuals who died without evidence of neurological or psychiatric disorder. The brains were removed 24–36 h after death, and each brain was divided in half down the mid-line. The right half was used for biochemical analyses, whilst the left half was fixed in formalin for neuropathological examination. Alzheimer's disease was confirmed histologically. There was no evidence of cerebrovascular disease in any of the thirteen cases. The ten controls ranged in age from 46 to 74; the Alzheimer patients were aged 61, 70, and 75. Tissues from the right half of the brain were stored at −190°C. Choline acetyltransferase (C.A.T.) and acetylcholinesterase (A.C.E.) activities were measured by the methods of Fonnum,[1] and glutamic acid decarboxylase (G.A.D.) activity by the method of Roberts and Simonsen.[2]

C.A.T. activity in the Alzheimer's disease brains was much reduced in the amygdala, hippocampus, and cortex (table I). Only three Alzheimer brains have been studied but the extent of the reduction in these areas strongly suggests this is not a chance occurrence. The activity of A.C.E. is dramatically reduced in the same areas of the cerebral cortex that show reductions in C.A.T. activity (table II), and is below the levels found in the normal brains in all the other areas. The areas of the cerebral cortex which show the maximum reductions in C.A.T. and A.C.E. activity are those which contain the greatest density of neurofibrillary tangles.

The reductions in the activity of the enzymes involved in the metabolism of acetylcholine are not a result of non-specific degenerative process. The activity of G.A.D. in all the areas of the Alzheimer's disease brains studied appears to be well within the normal range, means ranging from 74% to 121% of control activities. That this is the case in the cortical areas which show large losses of C.A.T. and A.C.E. supports the notion that a selective degenerative process has occurred. The normal values obtained for G.A.D. are of special significance because this enzyme is particularly sensitive to ante-mortem hypoxia.[3] It seems unlikely, therefore, that the decreased activity of enzymes associated with cholinergic transmission can be ascribed to this cause; none of the patients with Alzheimer's disease had prolonged terminal hypoxic episodes.

Preliminary studies of tyrosine hydroxylase, aromatic amino-acid decarboxylase, dopamine-β-hydroxylase, and monoamine oxidase indicate no loss of these enzyme activities in Alzheimer's disease and lend weight to the notion that a selective destruction of the cortical cholinergic system is an important feature of this condition.

We considered the possibility that selective loss of cholinergic system components could be due to prolonged drug regimens used in the patients with Alzheimer's disease. However, there is no standard drug therapy for Alzheimer's, and treatment is symptomatic. All three patients were given nitrazepam, but this drug was also given to five of the ten control patients during their terminal illness. Opiates were administered to two of the Alzheimer patients and five of the controls, and phenothiazines were given to one and two patients, respectively. Thus no drug treatment was exclusive to the Alzheimer's disease patients, and it seems improbable that the deficit in C.A.T. and A.C.E. activity in the cortex of these individuals is drug induced.

Expression of results relative to protein, D.N.A., or R.N.A. content does not alter the pattern of the results significantly.

If these data can be confirmed in a larger series of cases the concept of Alzheimer's disease as a cholinergic system failure may have important consequences for research on this condition.

TABLE I—CHOLINE ACETYLASE (μ mol/h/g WET WEIGHT)

Brain area	Controls (mean ± S.E.M.) (10)	Alzheimer's A	B	C	% of control (mean)
Hippocampus	0·7±0·2	0·06	0·05	0·06	8·1
Amygdala	2·0±0·4	0·08	0·3	0·2	9·6
Parietal cortex	0·5±0·1	0·15	0·1	0·07	21·0
Sensory cortex	1·0±0·1	0·03	0·1	0·08	7·0
Occipital cortex	0·5±0·1	0·05	0·1	0·1	16·6
Frontal cortex	0·7±0·1	0·2	0·4	0·1	33·3
Caudate	4·1±0·4	1·3	1·7	2·6	45·5
Substantia nigra	2·5±0·4	0·8	1·2	0·2	73·3
Mid-brain	1·1±0·2	0·3	0·5	0·3	33·3
Pons	1·4±0·3	0·9	0·6	0·3	42·8

TABLE II—ACETYLCHOLINESTERASE (mmol/h/g WET WEIGHT)

Brain area	Controls (mean ± S.E.M.) (10)	Alzheimer's A	B	C	% of control (mean)
Hippocampus	0·4±0·1	0·05	0·03	0·05	10·8
Amygdala	0·6±0·15	0·10	0·06	0·07	12·7
Parietal cortex	0·5±0·1	0·03	0·02	0·02	4·6
Sensory cortex	0·4±0·1	0·05	0·04	0·05	11·6
Occipital cortex	0·4±0·2	0·05	0·10	0·04	15·8
Frontal cortex	0·5±0·1	0·10	0·10	0·06	17·3
Caudate	1·75±0·2	1·0	1·1	0·9	57·1
Substantia nigra	0·75±0·1	0·15	0·2	0·1	20·0
Mid-brain	0·55±0·1	0·1	0·08	0·08	15·7
Pons	0·55±0·15	0·25	0·15	0·1	30·3

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HEADACHE AFTER LUMBAR PUNCTURE

SIR,—The frequency of headache after lumbar puncture in the four large series cited by Wolff was 25%.[1] The headache is thought to be due to continued leakage of cerebrospinal fluid (C.S.F.) through the hole in the theca, the subsequent low pressure in the C.S.F. pathways inducing pain by traction on the pain-sensitive neural endings in the dura and intracranial venous sinuses and arteries. Aqueous vasopressin injection ('Pitressin') as a prophylactic measure was popular some years

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Alzheimer's disease and Down's syndrome: roles of APP, trophic factors and ACh

Ole Isacson, Hyemyung Seo, Ling Lin, David Albeck and Ann-Charlotte Granholm

Recent therapeutic investigations of Alzheimer's disease (AD) have been guided by two seemingly opposed hypotheses: the amyloid cascade theory, which favors the amyloid plaques as the cause of AD; and the cholinergic theory, which favors cholinergic neuron loss as the cause. New investigations indicate that the synthesis and processing of the amyloid precursor protein (APP) is linked to the trophic actions of nerve growth factor. A pathological cascade in both AD- and Down's syndrome-related memory loss could be triggered by alterations in APP processing or ACh-mediated neuronal function, or both, which in turn trigger the overexpression of amyloid β, synaptic malfunction and trophic factor loss in target regions. This eventually leads to synaptic and dendritic loss with age.

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In Alzheimer's disease (AD)- and Down's syndrome (DS)-related dementia, the progressive nature of neurodegeneration suggests an age-dependent process that ultimately leads to degeneration of synaptic afferent systems, dendritic and neuronal damage, and the formation of abnormal protein aggregates throughout the brain. By the fourth decade of life, individuals with DS display many of the same neuropathological features (i.e. neuritic plaques, neurofibrillary tangles and degeneration of basal forebrain cholinergic neurons) as do individuals with AD, and many of these individuals develop dementia early in life [1–5]. It has been suggested that the neurodegenerative processes in AD and DS are closely related and at least partially comparable [4].

Recent findings indicate that cortical and hippocampal cholinergic synaptic systems [6,7] and trophic factors [8] can either reduce or accelerate pathogenesis and progression of AD and DS pathology by effects on amyloid precursor protein (APP) levels, metabolism and processing. The pathology of AD is determined diagnostically by several standardized clinical and pathological criteria [9–12]. Cortical plaques, neuronal tangles and degeneration of afferents to areas such as the hippocampus and neocortex are neuropathologically linked in a multifactorial progression. The exact physiological sequence of events leading to these pathologies is unknown.

The early discovery of ACh deficiency [13] singled out the loss of this neurotransmitter as one reason for the cognitive dysfunction in AD. Recent reports have detected only slight biochemical enzyme loss in the cholinergic system in mild cognitive impairment, which is thought to be the precursor to AD [14–17], although these studies have not determined whether the function of the cholinergic system is intact. Many elegant studies support the cholinergic hypothesis [18,19], showing that a dysfunctional cholinergic system is sufficient to produce memory deficits in animal models that are analogous to Alzheimer's dementia. Nevertheless, to date, little information exists about the dynamic processes that underlie the cognitive loss and neuropathology.

The cholinergic system (and potentially other afferent systems such as the noradrenergic and serotonergic systems), APP and trophic factors are intimately linked in normal function and possibly also in pathogenesis. This article provides a synthesis of these pathophysiological interactions and new interpretations of available data and recent findings.

Transgenic, mutant and animal models

Recently, a transgenic mouse has been described that expresses a neutralizing monoclonal antibody against nerve growth factor (NGF) [8]. In these mice, brain pathology exhibits remarkable similarities to the pathology seen in progressive AD, including amyloid plaques, hyperphosphorylated tau, neurofibrillary tangles in cortical and hippocampal regions, and marked cholinergic neuron degeneration. The anti-NGF transgenic mice show more pathology resembling that found in individuals with AD than transgenic mice that express mutant APP [8]. In fact, studies using the transgenic mice with human mutations of presenilin or APP, or both, have failed to demonstrate significant alterations in the cholinergic cell body region, even though in the APP–presenilin double mutant (APPK670N, M671L and PS1M146L [20]) there is a decrease in the density of cholinergic synapses in the frontal cortex [21].

Another study (using the human APP overexpression mutant, V717F [22]) also failed to detect significant degeneration of the cholinergic neurons, even at 18 and 26 months of age, despite significant levels of amyloid plaques in the brain. These findings from transgenic mice indicate that there are factors other than faulty amyloid processing involved in the neuropathology of AD.

Consistent with the findings using anti-NGF transgenic mice [8] described above, a toxic antibody (anti-NGF) treatment paradigm in rats leads to deterioration of cholinergic CNS basal forebrain neurons and synapses, and produces a shift towards APP elevations and decreased levels of choline acetyl transferase (ChAT), which are associated with behavioral deficits in spatial orientation and memory

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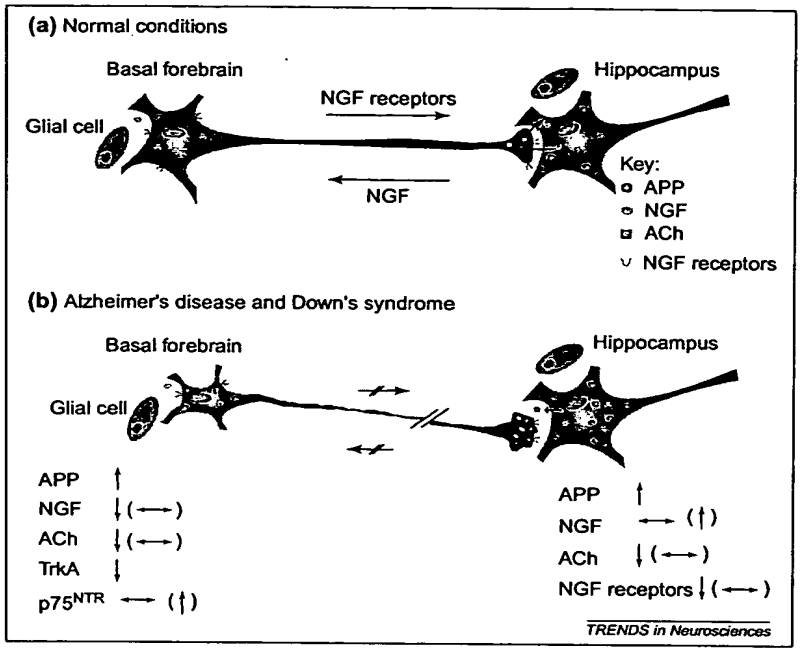

Fig. 1. Cholinergic basal forebrain neurons (a) in normal physiological conditions and (b) as postulated in individuals with AD and DS. In cholinergic neurons of the basal forebrain in individuals with AD and DS, ChAT immunoreactivity, cell size and number, and NGF and TrkA levels are decreased. In the hippocampus of individuals with AD and DS, ChAT levels and ACh-mediated signaling are reduced but NGF levels are increased or unchanged. In the hippocampus and basal forebrain of individuals with AD and DS, APP expression and Aβ aggregate levels are increased, whereas secreted (trophic) sAPPs are decreased. There are also degenerating terminals in the hippocampus. These altered levels indicate that NGF retrograde transport or NGF binding to trkA receptors, or both, are reduced in the individuals with AD, which results in inappropriate trophic support of the cholinergic system during degenerative disease [78]. Abbreviations: Aβ, amyloid β; ACh, acetylcholine; AD, Alzheimer's disease; APP, amyloid precursor protein; ChAT, choline acetyl transferase; DS, Down's syndrome; NGF, nerve growth factor; sAPP, soluble APP; TrkA, tyrosine receptor kinase A.

tasks [6,23]. In addition, studies have been performed on mice with a segmental trisomy of chromosome 16, Ts65Dn [24,25]. The triplicated gene segment includes the gene for APP, and other genes that are present in individuals with DS [26]. These mice

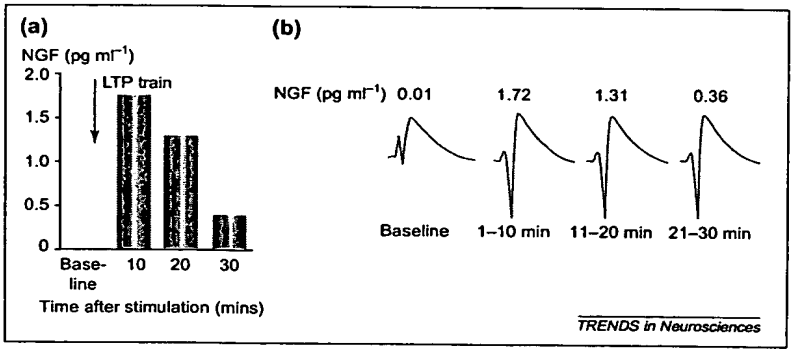

Fig. 2. Long-term potentiation (LTP)-induced nerve growth factor (NGF) release in a hippocampal slice from a young rat. (a) LTP was induced by rapid-train stimulation with an electrode, and NGF was measured by a specific NGF enzyme-linked immunosorbent assay kit in the superfusate after protein purification at different times after stimulation (10, 20 and 30 min). (b) The field potentials show the LTP induction and the NGF concentration in pg ml^{-1}. These data suggest a dynamic interaction between hippocampal activity and NGF release that could regulate synaptic terminal growth and function [67,68]. These regulatory release patterns were later also confirmed for another member of the neurotrophin family, brain-derived neurotrophic factor (BDNF) [69].

overexpress full-length APP and amyloid β, and undergo significant deterioration of the cholinergic system and basal forebrain NGF levels as young adults [24]. Ts65Dn mice exhibit reductions in basal forebrain cholinergic neuron size and number, and regressive changes in the hippocampal terminal cholinergic axonal fields, which are thought to be associated with impaired retrograde transport of NGF from hippocampus to the basal forebrain [27,28].

All of these recent animal models re-emphasize the possibility that pathological processes operating in age-related cognitive impairment, idiopathic AD and DS might be related to physiological changes in several systems, including synaptic complexes associated with cholinergic nerve terminals. Such synapses depend on the trophic action of NGF for their function [29–32]. As cholinergic degeneration in individuals with AD is closely correlated with the degree of memory impairment [13], this transmitter system and related trophic factors might also be associated with amyloid plaque formation and related trophic factors, neuronal tangles and cell degeneration (Fig. 1).

APP-related systems interact with both trophic factors and cholinergic receptors

A high percentage of basal forebrain cholinergic neurons degenerate in AD [5]. The cholinergic neurons are highly dependent upon NGF for their function [33–36], and use high-affinity tyrosine receptor kinase A (trkA) and low-affinity (p75) receptors for signaling. The trkA receptors are synthesized in cholinergic neurons and are necessary for binding and retrograde transport of the growth factor from the terminals to the cell body region, while the low-affinity receptors appear to be important for sequestering growth factor molecules to the presynaptic membrane [37–40]. However, NGF is synthesized by neurons in the target regions and is released in an activity-dependent manner [35,41,42] (Figs 1–3).

In individuals with AD, there is typically a marked loss of high-affinity trkA receptors in both cholinergic target neurons and basal forebrain neurons, which correlates with loss of cholinergic neurons [43–45]. Even though there have been some variable results regarding NGF protein levels in different brain regions of individuals with AD, most recent studies agree that there are unchanged or increased NGF levels in the hippocampus and cerebral cortex, while the levels in the basal forebrain are decreased compared with age-matched controls [46–53]. This, together with cholinergic neuron loss and dysfunction, suggests that NGF is not adequately transported retrogradely to the basal forebrain or that binding to the high-affinity receptors or release of NGF from hippocampal interneurons, or both, is deficient in the brain of individuals with AD and in animal models of aging (Figs 1,3) [49,54].

Infusion of NGF can prevent degeneration of axotomized cholinergic CNS neurons [55]. A biological basis for an interaction between APP and NGF has

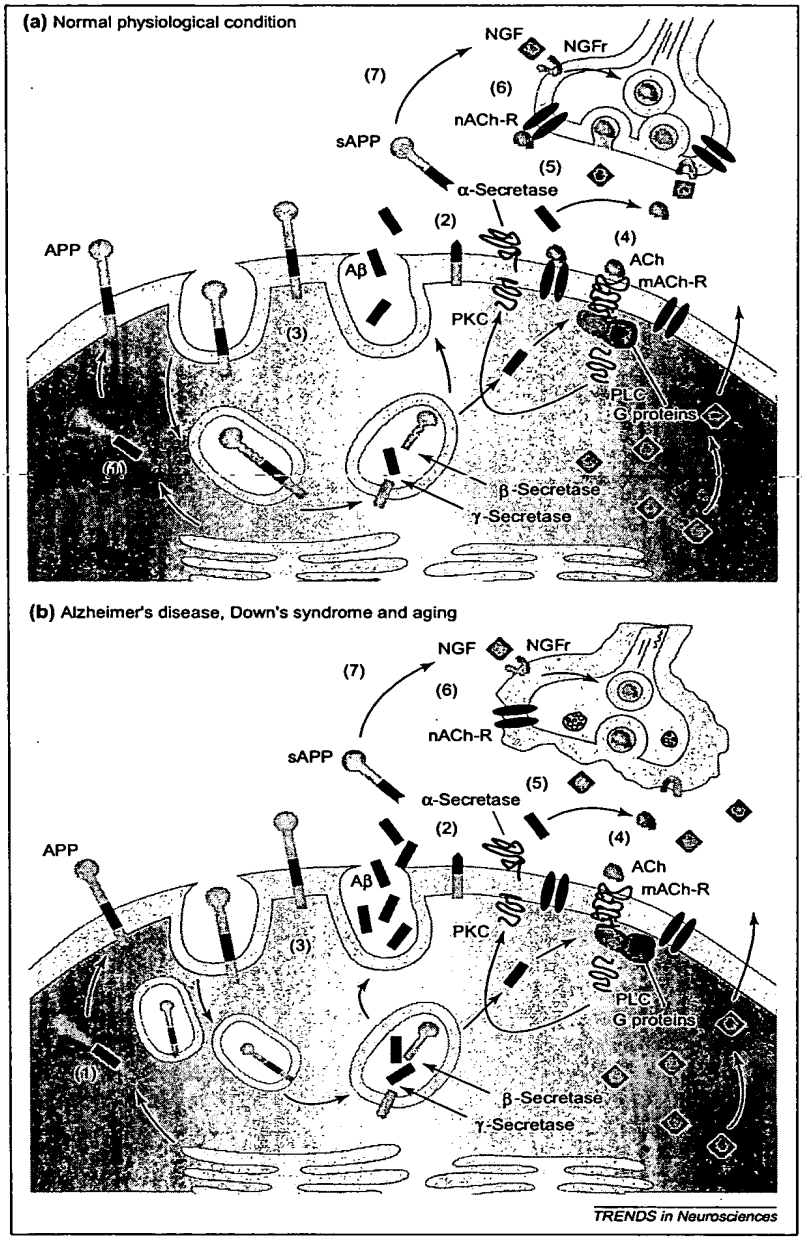

Fig. 3. Different pathways for amyloid precursor protein (APP) processing and nicotinic-receptor- and muscarinic-receptor-mediated regulation of APP metabolism (nACh-R and mACh-R, respectively). (a) (1) Newly synthesized APP is transported from the Golgi through vesicles to the cell surface (2) where it can be cleaved within the amyloid β (Aβ) domain by α-secretase. Mature cell-surface APP can also be reinternalized (3) into late endosomes and lysosomes, where it can be processed by β-secretase and γ-secretase to yield Aβ, which can be rapidly secreted into the extracellular fluid. (4) Acetylcholine (ACh) is released because of the arrival of an action potential. It binds to α7 nicotinic receptors (α7 nACh-R) presynaptically, and α7, M_1 and M_2 receptors postsynaptically. The α7 nACh-R binding results in presynaptic Ca^{2+} influx, which leads to increased ACh-mediated tone. Released ACh from presynaptic terminals also binds to G-protein-coupled muscarinic receptors and subsequent activation of phospholipase C (PLC), which hydrolyzes phosphoinositolipids to inositol-3-phosphate and diacylglycerol. Diacylglycerol activates protein kinase C (PKC), which directly or indirectly enhances α-secretase-mediated cleavage of APP. (5) Aβ can interfere with cholinergic neuron function at both presynaptic and postsynaptic signaling sites (4) and (5), and increased ACh-mediated signaling leads to decreased APP levels and increased release of soluble APP (sAPP). (6) Acetylcholine release increases NGF secretion from the postsynaptic membrane, which binds to presynaptic NGF receptors (NGFr), resulting in retrograde transport of NGF to the cell body of cholinergic neurons. (7) The signaling of NGF to NGF receptors is stimulated by sAPP. (b) In Alzheimer's disease and Down's syndrome, pathological conditions and aging, there is damage to terminals and (1) increased APP expression, (2) increased Aβ secretion by amyloidogenic processing, (3) internalization of mature cell-surface APP, (4) reduced ACh neurotransmission, (5) increased Aβ interference of ACh signaling, and (6) reduced transport of NGF–NGFr complexes to the ACh cell body.

been suggested by cell culture studies that demonstrate a dose-dependent relationship between NGF and APP messenger induction [56–58]. After exposure to NGF, primary cortical neuronal cultures showed increased levels of membrane phospholipids that might promote APP expression and secretion of the soluble form of APP (sAPP). The large membrane-spanning precursor molecule (full-length APP) can be processed into several different biologically active compounds, such as the secreted form, sAPP, which has been shown to have neurotrophic activities, and the longer aggregating forms, of which amyloid β_{1-42} is the most toxic [53,58,59] (Figs 1,3). Cell culture work also indicates that sAPP potentiates the effects of NGF on differentiation of catecholaminergic cell lines [60]. Soluble APP can also augment the effects of NGF, and, conversely, the expression and release of APP is temporally enhanced by NGF *in vivo* [58]. A trophic relationship between sAPP and NGF has thus been established.

The cholinergic system has been shown to have a regulatory effect on both APP and NGF-related processes. Some types of cholinergic neuron lesions can increase hippocampal NGF levels in the normal adult rodent brain [61–63]. Other findings connect muscarinic-receptor agonists and other ACh-related agents with NGF and APP. For example, recent studies have demonstrated that amyloid β_{1-42} binds to α7 nicotinic ACh receptors with high affinity [64]. In the same study, it was also shown that the blocking effect of amyloid β_{1-42} on the presynaptic nicotinic ACh receptors gave rise to decreased Ca^{2+} influx, and thus inactivation of the presynaptic membrane (hence, a decreased ACh-mediated tone was produced by amyloid β_{1-42} administration).

On the basis of these and other recent *in vivo* findings using ACh M_1-receptor agonists [8,9,65], it is postulated that close relationships exist between the function of trophic factors, APP and neurotransmitters such as ACh in regulating the health of neurons (Figs 1,3). A pivotal pathological cascade in both AD- and DS-related memory loss might be triggered by alterations in APP processing or cholinergic neuronal dysfunction, or both, which triggers overexpression of amyloid β, synaptic malfunction and trophic factor loss in target regions (eventually leading to synaptic and dendritic loss in all regions involved). By this and other genetic mechanisms, abnormal levels of neurotrophic

sAPP and pathogenic levels of solubilized amyloid β might eventually cause progressive (and regressive) degeneration of cholinergic nerve terminal function in target regions (hippocampus and cerebral cortex), and thus decreased ACh-mediated tone, which leads to decreased NGF release and uptake, cholinergic neuronal cell body atrophy, and metabolic downregulation (Figs 1,3).

NGF, LTP and the cholinergic hippocampal synapse

Given that long-term potentiation (LTP) has been used as a model for synaptic plasticity and learning in the hippocampal formation, it is relevant to determine whether NGF is associated with LTP [64]. Indeed, evidence shows a significant increase in basal NGF release after LTP induction in hippocampal slice cultures (Fig. 2) [67,68]. These regulatory release patterns were later confirmed for another member of the neurotrophin family, brain-derived neurotrophic factor [69]. That hippocampal target levels of NGF are increased in aging and sometimes in AD [46], seems to contradict the degeneration and atrophy seen in cholinergic neurons, but is explained by disease and age-dependent mechanisms of reduced NGF uptake by cholinergic nerve terminals [49].

LTP induces an acute and specific release of NGF in the hippocampus, which is consistent with reports that ACh-receptor agonists have this effect [42]. However, chronic over-stimulation by ACh-receptor agonists *in vivo* in young adult mice leads to a compensatory reduction in hippocampal NGF levels, probably as a feedback signal to lower cholinergic nerve terminal function and growth (Fig. 1) [70]. Interestingly, local application of NGF to basal forebrain cholinergic neurons can give rise to a slow-onset (~20 min) but significant increase in basal firing rate, especially in cholinergic neurons in aged animals that have been shown to have decreased basal forebrain levels of NGF [68]. This increase in cholinergic neuron activity in response to locally administered NGF seen in aged animals might be an adaptive mechanism that recruits NGF to a system in need of trophic support. Thus, there is also a close relationship between NGF and this cholinergic pathway in rapid cellular events in the basal forebrain.

It is possible that this two-way relationship is altered by events that lead to AD and DS. Enhanced LTP and afferent synaptic strength via cholinergic, serotonergic and, possibly, noradrenergic systems in the hippocampal formation has been shown to enhance memory function. Conversely, impairment of these transmitter systems reduces LTP and memory function, at least in hippocampal-dependent tasks [24,71]. As previously discussed, this is modeled by transgenic expression of an antibody against NGF [8]. Such an antibody or toxin to the trkA receptor [6,23] selectively disrupts the ACh-mediated innervation of hippocampal neurons and the functional integrity of this system. Further evidence of a close relationship between ACh-mediated transmission and APP, is the increase in the levels of sAPP found after M_1-receptor activation. Soluble APP is considered to be a trophic substance in these systems [72–77]. Increased NGF levels seen in our studies after LTP (Fig. 2), combined with an increased release of sAPP under maximal activation of the cholinergic system would enhance NGF function. In any case, these intracellular changes and reduced metabolic functions are consistent with a dysfunction of the cholinergic system and trophic target zones in the hippocampal formation, and of other ACh-receptive regions of the cerebral cortex (Fig. 3) [65].

Comprehensive AD- and DS-like pathologies are thus produced by altered cholinergic and APP-related systems in trisomic mice that overexpress APP [27] or by *in vivo* NGF dysregulation via antibodies directly against NGF or its receptors [6,8,65]. Recent observations are consistent with the idea that there are homeostatic mechanisms regulating hippocampal NGF, APP and ACh-mediated activity. A dysregulation of one or more of these three factors would progressively lead to imbalance in neurotransmission, eventually leading to synaptic damage and neuronal cell loss relevant to memory function. Models that focus on complex interactions involved in dementias might be more realistic for achieving new drug, cellular and molecular therapies to influence both stage- and age-dependent neurodegenerative disease.

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A rationale for the structure of color space

R. Beau Lotto and Dale Purves

The colors perceived by humans in response to light stimuli are generally described in terms of four color categories (reds, greens, blues and yellows), the members of which are systematically arrayed around gray. This broadly accepted description of color sensation differs fundamentally from the light that induces it, which is neither 'circular' nor categorical. What, then, accounts for these discrepancies between the structure of color experience and the physical reality that underlies it? We suggest that these differences are based on two related requirements for successful color vision: (1) that spectra be ordered according to their physical similarities and differences; and (2) that this ordering be constrained by the four-color map problem.

The goal of any visual system is presumably to distinguish physically different objects and the conditions under which they are witnessed, thus enabling successful visually guided behavior. All visual animals achieve this end by detecting differences in the quantity of the LIGHT (see Glossary) reflected by objects or otherwise returned to the eye, which are perceived in humans as LIGHTNESS and/or BRIGHTNESS. Many animals also distinguish objects according to differences in the quality of the light they reflect (i.e. the distributions of the spectral power in the stimulus), which are perceived in humans as different COLORS.

Although descriptions of the organization of human color sensations differ in detail [1–4], they share several key features. Thus, at any given level of light intensity, color experience can be portrayed as a plane in which movements around the perimeter correspond to changes in hue and movements along its radial axis correspond to changes in saturation (i.e. changes in the relative grayness of the color) (Fig. 1). In contrast to the continuously variable spectral distributions that generate sensations of color, all colors are experienced as belonging to one of four perceptual categories (reds, greens, blues and yellows), or combinations thereof. Thus, although the relationships between other visual sensations and the physical world that gives rise to them (e.g. sensations of shape, depth and motion) appear straightforward (i.e. the structure of physical space is roughly similar to the overall structure of the perceptual space it generates), color sensations are different: there is no obvious basis in the physical characteristics of light for either the circular ordering of colors in a plane, or their parcellation into four perceptual categories.

Why, then, is perceptual color space structured in this way, and does this structure have deeper implications for understanding the nature of vision generally? To the extent that contemporary theories of color vision have addressed these questions, the subjective structure of color experience is considered an inevitable consequence of TRICHROMACY and OPPONENCY ([5–9], but see Ref. [10]). Thus, most modern work has understandably focused on determining the cellular bases of these two physiological pillars of color sensation. As a result, the rationale for the structure of color experience is, in this view, secondary to the evolutionary value of trichromacy and opponency as such. Some of the advantages that have been suggested are: (1) optimally satisfying the constraints of information theory [11–13]; (2) promoting the perception of 'color constancy' [14–16]; and (3) helping our frugivore ancestors detect ripe fruit [17,18].

We take the opposite approach to understanding color experience. Rather than rationalizing the structure of color sensations in terms of trichromacy and opponency, we consider the structure of color space itself, asking whether color space (and thus the physiology that generates it) might represent the solution to the two fundamental problems in topology with which the evolution of color sensations must ultimately contend.

Distinguishing territories by spectral information

In examining the proposition that the structure of color experience, as such, should be a better guide to understanding color vision, a good place to start is to consider why color sensations have evolved in the first place. Many animals do not have a significant degree of color vision, and even those that do are for the most part more limited in color perception than are

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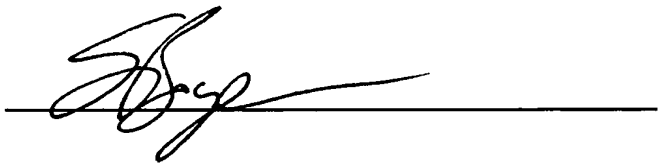
IN THE MATTER OF
US Serial No: 09/147,490
entitled"Neuroactive Peptide"

EXHIBIT 6

This is Exhibit 6 referred to in Clause 13 of the Statutory Declaration Siew Yeen Chai dated 13th Day of January 2004.

Before me: 

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Current Trends Review

Effects of Anticholinergic Drugs on Learning and Memory

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ABSTRACT

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Declines in cognitive function during normal aging and in dementic disorders have frequently been hypothesized to involve a decline in cholinergic transmission in the brain. Muscarinic cholinergic receptor blockade produces behavioral effects in animals reminiscent of aging-related performance deficits. The purpose of the present review is to analyze the results of previous behavioral experiments with antimuscarinic drugs in order to generate an hypothesis of the cognitive effects of muscarinic blockade. Given this hypothesis, it is suggested that experiments may be designed in which the antagonism of

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antimuscarinic drug effects may be used in the development of geriatric and antidementic drugs.

Key words: scopolamine, atropine, learning, memory, animals, geriatric model

INTRODUCTION

Due to the ever-increasing percentage of the aged in the population and the increasing number of people with dementic disorders, attention is being focused on the development of drugs for the treatment of the cognitive decline observed during aging and dementia. This pursuit can be aided by the availability of animal models and/or chemical means of reproducing dementia-like symptoms experimentally. In this regard, anticholinergic drugs have often been used to cause deficits of learning and memory. The work with anticholinergic drugs gathers further importance with the many recent proposals that dysfunctions of brain cholinergic pathways are critical in the development of presenile and senile dementia. The purpose of this review is to examine more critically a number of key experiments based upon which conflicting claims were made regarding the cholinergic involvement in learning and memory. In so doing, an attempt will be made to develop an hypothesis of the cognitive effects of anticholinergic drugs that is consistent with the reviewed data.

Antimuscarinic drugs have long been known to disrupt learned behaviors in rather specific ways. Several different theoretical formulations of cholinergic function have been developed focusing primarily on concepts such as behavioral inhibition [Carlton, 1963], discrimination [Milar et al., 1978], learning [Deutsch, 1973], and memory [Bartus and Johnson, 1976]. Their differences seem to be due to differential emphases on various groups of experimental findings. In this review, we will examine several experiments in an effort to demonstrate the importance and usefulness of discerning the mode of interaction between anticholinergic drugs and learning and memory processes. In so doing, we selected to focus largely on muscarinic receptor antagonists for two reasons. First, the vast majority of acetylcholine (ACH) receptors in the mammalian brain are of the muscarinic type. The predominance of muscarinic receptors is also apparent in brain structures that seem specifically involved with attentional, acquisitional, or memorial function, such as the hippocampus. Second, the effects of systemic muscarinic blockade on performance in memory tasks are much more striking than those of nicotinic stimulation or blockade.

Much of the interest on the effects of anticholinergic drugs concerns whether these effects are directly involved with the acquisition (learning) and/or maintenance (memory) of associative links between stimuli or between stimuli and responses or whether they affect cognitive processes that are only indirectly involved in learning and memory. Such attendant processes that have been thought to be affected include perceptual discrimination, selective attention, and overall excitatory–inhibitory response "tone" (the general tendency to emit or withold a response). Significant effects in any one of these realms could produce a deficit in the performance of many behavioral experiments. Accordingly, we will review and summarize the effects of antimuscarinic drugs on animals performing different types of associative tasks. Drug effects on the development (learning) and maintenance (memory) of associative strength will be estimated through their effects on stimulus control: i.e., the degree of relationship between experimental variations in conditioned or discriminative stimuli and variations in the subsequent response accuracy.

In discussing the evidence on the actions of anticholinergic drugs, data will be presented in procedurally defined units, starting with the classical conditioning paradigm. Spontaneous alternation (proposed by Douglas and Isaacson [1966] to be based on habituation), passive avoidance, and conditioned suppression will be considered next, followed by active avoidance, discrimination, and memory procedures. Memory procedures will be split into two main categories: delayed response and delayed conditional discrimination. In the former, the correct response to be made at the end of the retention interval is specified by a stimulus presented

before the interval. In the latter, the type of correct response is unspecified until a comparison stimulus is presented at the end of the retention interval. The response is, however, usually specified in terms of the stimulus (e.g., go to the red).

ANTIMUSCARINIC DRUGS

Although several new drugs have been developed that interact with the muscarinic receptor in various ways and with various affinities, the discussion in this review will focus on the effects of two drugs: scopolamine and atropine. The reason for this selectivity is simply that very little research has been done on the behavioral effects of other agents. Both scopolamine and atropine bind to the muscarinic receptor in the low nanomolar range. However, the degree of specific binding of scopolamine is even higher than that of atropine: only 2.2% of bound scopolamine is nondisplaceable [Hulme et al., 1978]. Thus, although these agents have been known and used for over a century, they are still among the most potent and specific muscarinic antagonists known.

Methscopolamine bromide and atropine methylnitrate are quaternary ammonium derivatives of scopolamine and atropine, respectively. Since they are more polar than their parent compounds, they have a more limited ability to penetrate into the central nervous system. The largely peripheral effects of these quaternary agents has thus made them suitable for use in determination of the contribution of peripheral muscarinic receptor blockade to the behavioral effects of scopolamine and atropine. In most behavioral paradigms purporting to measure learning and memory, the quaternary ammonium compounds have much less effect than the same doses of the unaltered belladonna alkaloids.

DRUG EFFECTS ON LEARNING AND MEMORY-RELATED BEHAVIORS

Classical Conditioning

In classical (Pavlovian) conditioning, a previously neutral stimulus such as a tone is presented immediately before a stimulus that unconditionally provokes a response from the organism (e.g., presentation of food, resulting in a salivation response). After a number of such pairings, the preceding neutral stimulus begins by itself to evoke a response usually quite similar to that produced by the unconditioned stimulus. The first systematic investigation of muscarinic antagonists on the acquisition, maintenance, and extinction of classical conditioning was performed by Downs and co-workers [1972]. Using the rabbit nictitating eye membrane (NM) preparation, shock to the outer eyelids was the unconditioned stimulus (US) for NM extension (the unconditioned response, UR) while tones of varied frequency served as either the conditioned stimulus correlated with shock (CS+) or that uncorrelated with shock (CS−). Injection of atropine sulfate (a muscarinic receptor blocker), but not of atropine methylnitrate (a quaternary ammonium form of atropine that does not readily pass the blood–brain barrier) or saline, retarded the rate of acquisition of conditioned responses to the CS+ in doseages from 10 to 26 mg/kg. However, the frequency of URs throughout acquisition was uninfluenced by the drug. Atropine was also found to reduce or abolish the occurrence of conditioned heart rate changes in response to the CS+ and CS−. When a separate group of rabbits first received extensive conditioning with no drug and were then exposed to it after asymptotic conditioning had occurred, atropine sulfate, but not atropine methylnitrate or saline, markedly decreased conditioned NM responses (CRs) to the CS+ and did not affect CRs to the CS−, which were almost nonexistent under control conditions. Finally, these experimenters conditioned previously untreated rabbits and extinguished the conditioning (presented CS+s and CS−s without the USs) either under atropine sulfate, atropine methylnitrate, or saline. Although atropine sulfate reduced the percentage of CRs to both CSs in the first two extinction sessions as compared to either control treatment, when saline was administered on the third extinction session, the percentage of CRs was no different from that during asymptotic conditioning

sessions. These data indicate that while central muscarinic antagonists did not affect expression of the NM response itself (since percentages of URs were unaffected), these drugs did retard acquisition of stimulus control by CS+ and CS− as well as extinction of stimulus control. Moreover, atropine disrupted the expression of a previously conditioned response to the CS+.

Moore et al. [1976] also used classical conditioning of NM response to investigate the effects of another muscarinic antagonist, scopolamine hydrobromide. In their first experiment, these investigators found that scopolamine (1.5 mg/kg) failed to affect expression of the UR to a low-intensity US (0.25 mA), thus agreeing with Downs et al. [1972]. In addition, scopolamine did not affect habituation to the US. Also in agreement with Downs et al. were their findings that scopolamine hydrobromide (but not scopolamine methylbromide, a form that does not readily penetrate the blood–brain barrier) markedly retarded acquisition of the CR. In an effort to discover whether central muscarinic blockade affected registration of the CS ("input" processes), Moore and co-workers determined the threshold intensities of the tonal CS in producing a CR. While scopolamine hydrobromide did increase the CS threshold intensity from 48 to 58 dB SPL, thresholds were still lower than the CS intensity used in conditioning (75 dB SPL). In order to determine whether these effects were specific to auditory CSs, acquisition of CRs to visual CSs and corresponding CS threshold intensities were studied. Although scopolamine hydrobromide again retarded acquisition, no significant increase in visual CS threshold intensity was noted. Moore et al. attributed this auditory–visual threshold discrepancy to the fact that several cholinergic pathways are known to exist in regions of the central nervous system known to be involved in auditory processing.

Most recently, Harvey et al. [1983] have reported a thorough analysis of the effects of scopolamine hydrobromide and methylbromide of classical conditioning of the rabbit NM response, using both auditory and visual CSs. These authors examined a wide range of scopolamine doses (0.005–1.6 mg/kg, i.v.) and tried to define more precisely the behavioral mode of action. They first supported previous findings by showing that scopolamine retarded acquisition at doses not affecting the UR or threshold intensity of the US. In addition, scopolamine was again found to increase the auditory CS intensity necessary to produce a CR. Finally, Harvey et al. demonstrated that the retardation of acquisition produced by several preexposures to the CS alone was not affected by scopolamine. Therefore, scopolamine did not appear to produce its effects through interfering with other nonassociative processes, such as "the development of habituatory decrements during unpaired stimulus presentations" (i.e., latent inhibition). This demonstration of a lack of effect by scopolamine on the development of latent inhibition also indicates that scopolamine could not have blocked the registration (or "unconditioned" excitatory effects) of the CS.

Taken together, the data presented above lead to the following conclusions on the effects of antimuscarinic drugs. First, the motoric expression of the UR ("output") is unaffected. This is unlikely to be due to the possibility that UR expression was at ceiling or that the US was far suprathreshold since Moore et al. [1976] found the same result using low-intensity USs. Second, although the registration ("input") of auditory CSs may be reduced, this effect is certainly not strong enough to account for the reduction in the acquisition and maintenance of stimulus control by the CS produced by antimuscarinics since 1) the latent inhibition experiment by Harvey et al. indicate unimpeded stimulus registration, and 2) although visual CS thresholds are unaffected, scopolamine produced the same reduction in associative stimulus control by visual CSs as by auditory CSs. Third, nonassociative changes in the unconditioned excitation produced by CSs (latent inhibition) and USs (habituation) are unaffected. Fourth, Carlton [1963] and others have proposed that antimuscarinics chiefly interfere with behavioral inhibition—the process mediating suppression of punished or extinguished responses. If behavioral inhibition was involved in suppressing CRs to the CS− in either the Downs et al. [1972] or the Moore et al. [1976] study, one would expect either atropine or scopolamine to increase the occurrence of CRs to the CS−. Such was not observed to be the case. Fifth, Milar et al. [1978] hypothesized that antimuscarinics disrupt the discriminatory process—the ability to tell stimuli apart and differentiate responses to them. Thus, if the discriminatory process were impaired in the absence of any changes in overall behavioral excitation/inhibition, CRs to the

CS+ would be expected to decrease and CRs to the CS-- would be expected to increase. Again, this pattern of results was not observed. Thus, antimuscarinics would seem to produce their behavioral disruption by specifically interfering with the maintenance and perhaps even formation of the association between CS and US. It must be noted that the fact that antimuscarinics retard acquisition does not logically require that formation of associative links be interfered with; an ongoing interference with maintenance or expression of the forming association would suffice. Therefore, the most parsimonious working hypothesis of the effects of centrally acting antimuscarinics on aversive classical conditioning of the NM response in rabbits is that the maintenance or expression of associative links is disrupted. The cognitive equivalent of this hypothesis is that either retention or retrieval of previously formed associations is perturbed by central muscarinic antagonist drugs.

Spontaneous Alternation

In the spontaneous alternation (SA) paradigm, subjects are typically given two to three unrewarded trials per day in a T or Y maze. Untreated naive rats display a significant tendency to alternate arm choices over trials. The occurrence of spontaneous alternation has been shown to increase as a direct function of the time subjects are detained in the previously chosen arm [Kirkby et al., 1967] and to depend on olfactory and vestibular cues [Douglas, 1966; Rosen and Stein, 1969]. It has been hypothesized by Glanzer [1953] that SA is a function of satiation or habituation to previously encountered stimuli. However, it should be clear that habituation alone cannot account for SA; the habituated stimulus must also be associated with either the response of turning toward that stimulus or the olfactory stimulus paired with approaching that stimulus.

Several investigators have demonstrated that both atropine and scopolamine can potently decrease and physostigmine increase the rate of SA [Douglas and Isaacson, 1966; Leaton, 1968; Squire, 1969; Swonger and Rech, 1972; Drew et al., 1973]. The fundamental nature of the scopolamine disruption of SA is emphasized by the finding that even when genetic differences in mouse strains lead to opposite responses to scopolamine in activity and exploration (e.g., object sniffing and rearing), scopolamine uniformly disrupts SA [VanAbeelen and Strijbosch, 1969; Anisman, 1975; Anisman and Kokkinidis, 1975].

However, there is good evidence that scopolamine does not disrupt SA performance when additional stimulus information is presented during the trials. Leaton and Buck [1968] showed that when, on trial one in a T maze, one arm was black and one white and both arms became either black or white on trial two, rats entered the arm that was changed in color whether treated with saline or scopolamine. Similarly, Leaton and Utell [1970] showed that SA was unimpaired by scopolamine when on trial one, subjects were "forced" into one arm (a door covered the entry into the other arm). Since SA occurred even following scopolamine treatment in these cases, it could be argued that the effects of antimuscarinics on SA reported in the preceding discussion are due to a partial disruption of either habituation or retention/retrieval of the associative link between the previously chosen arm and the corresponding odor or response. Defects in formation or acquisition of the association cannot be the sole effect produced by the drug, since Squire [1969] demonstrated that physostigmine and scopolamine had significant and opposite effects when given either before or after trial one. However, both drugs had less effect on SA when given 60 or 120 min after trial one. Squire interpreted this finding as indicative of an effect on "consolidation" of memory. The next section will present more evidence on putative consolidation effects.

Passive Avoidance

The passive avoidance (PA) procedure has two main variations. In the first, called "step-through" avoidance, subjects are placed into one compartment of a chamber and access to another compartment is allowed. Once the subject enters the second compartment, a door is closed and the floor bars are mildly electrified. The second is called "step-down" avoidance. Subjects are placed on a platform above floor bars in a chamber. When the subject steps down from the platform to the floor bars, the subject receives a mild foot shock.

PA is referred to as a one-trial learning procedure because one brief exposure (trial) to the paradigm is usually enough to produce significant increases in the latency to step-through or step-down on a second trial as many as 7 days later. As such, the procedure is well suited for evaluating potential drug effects on the consolidation of memory for the training trial.

When either scopolamine or physostigmine is administered before the first trial, trial two response latency is reduced [Buresova et al., 1964; Meyers, 1965; Bohdanecky and Jarvik, 1967; Glick and Zimmerberg, 1972; Hamburg and Fulton, 1972]. While Meyers [1965] found that scopolamine given just prior to a tenth training session produced a retention deficit, Buresova et al. [1964] found no such retention deficit when they administered atropine before trial two.

Another area of controversy lies in the effects of cholinergic drugs on consolidation when given immediately after the first (training) trial. Glick and Zimmerberg [1972] demonstrated that, when given at this time, scopolamine hydrobromide (and not methylscopolamine hydrobromide) disrupted retention test latencies 1, 2, and 7 days after training. However, Bohdanecky and Jarvik [1967] found no such effect. Both studies employed a one-trial step-through procedure in which mice were placed in a small, brightly lit compartment and were allowed access to a large, dark compartment. The only apparent difference was that the former study used a scopolamine dose of 10 mg/kg and the latter, only 1 mg/kg. However, 10 mg/kg of scopolamine is an extremely high dose for mice, and it is likely that some nonspecific effects were produced, perhaps accounting for the disagreement. At any rate, the lower dose was sufficient to produce large decreases in trial two latency when given before trial one. Similarly, using an atropine dose (6 mg/kg) high enough to produce a large trial two effect when given immediately before either trial one or trial two, Buresova et al. [1964] found no evidence for a consolidation effect.

Thus, the evidence supporting an effect of antimuscarinics on the so-called consolidation phase of memory in the PA procedure is inconclusive. Moreover, the elusiveness of the consolidation effect as compared to the inarguable effects of pretrial administration indicates that "consolidation disruption" is not the primary effect of cholinolytics. Furthermore, the demonstration by Hamburg and Fulton [1972] that physostigmine-induced "amnesia" in the PA procedure could be reversed by a brief reexposure ("reminder") to any facet of the task (the chamber, step-down platform, or shock alone), indicates that the deficit is a matter of retrieval, rather than retention. Indeed, the findings in the SA paradigm, that an increase in the number of or exposure to stimuli reduces the effects of cholinergic drugs, also indicates that disruption of retention is not the principal drug effect.

Taking the strong cholinergic effects on acquisition into account, it is apparent that a slightly more complex conceptualization is necessary. Antimuscarinics and anticholinesterases seem to exert the most disruption during acquisition and upon retest. Therefore, cholinergic drugs may disturb performance by interfering with the dynamic processes of encoding (storing in retrievable form) and retrieval of associations. Stated in terms of information processing theory, cholinergic drugs may distort input/output transfers to and from memory storage. This notion is consistent with the evidence presented to this point and excludes cholinergic drug effects on behavioral inhibition, habituation, memory storage, discrimination, and selective attention.

Conditioned Suppression

Berger and Stein [1969] examined the effects of scopolamine on mice in a one-trial conditioned suppression procedure, quite similar to PA. Subjects were trained to drink a fixed amount of water from a tube in a chamber with an electrifiable floor. Conditioning involved delivering a foot shock after drinking on trial one. Retention on trial two was measured by the latency required to consume that fixed amount of water. When scopolamine (1 mg/kg) was given before trial one, latency to drink on trial two was markedly reduced. When given solely before trial two, scopolamine had no effect when trial one shock was relatively intense (1 mA).

When trial one shock was reduced to 0.5 and 0.4 mA, pretrial two injection of scopolamine decreased the latency to drink. The same pattern of results over shock intensity occurred when scopolamine was given prior to both trials one and two.

Thus, Berger and Stein's data support the acquisition half of the encoding/retrieval hypothesis formulated in the last section. An interesting sidelight is the partial tendency towards state dependence in Berger and Stein's results. That is, under high shock conditions, scopolamine had no effect when administered before both trial one and trial two. This was not observed in the PA studies discussed previously, but is still not inconsistent with the current hypothesis. If scopolamine perturbed encoding on trial one in a certain way, it is conceivable that a similar perturbation in retrieval on trial two would aid performance. The current hypothesis does not demand this, however.

One-Way and Shuttlebox Avoidance

One-way avoidance typically involves placing subjects in one part of a two compartment chamber and providing a number of pairings between either an auditory or visual CS and a shock US. Subjects eventually learn to avoid the shock by crossing over into the opposite compartment when the CS predictive of shock is presented. Shuttlebox, or bidirectional avoidance is similar, except that animals are trained to shuttle back and forth over trials—the previous start box becoming goal box and vice-versa. Anisman [1973] examined the effects of scopolamine (1.0 mg/kg) and physostigmine (0.5 mg/kg) when injected before the second 50-trial session of either type of avoidance. In agreement with other reports, scopolamine was found to increase the number of avoidance responses in the bidirectional procedure but did not affect performance in the one-way task. Physostigmine reduced the number of avoidance responses in both tasks.

Baseline performance of one-way avoidance has often been found to be better than that in shuttlebox avoidance (as it was in Anisman's study) and the one-way task is acquired more rapidly. These findings have been ascribed to the theoretical consideration that in shuttlebox avoidance, the subject must repeatedly extinguish the association between shock and the compartment just departed from (developed often in acquisition, when an avoidance response is not emitted in time) in order to emit an avoidance response back to that same compartment. It is, therefore, understandable that scopolamine could improve avoidance performance by interfering with the encoding or retrieval of compartment-shock associations. Data from previous sections have shown that when associations are "overlearned," they are less vulnerable to disruption by antimuscarinics. Since nearly all acquisition trials contribute to the strength of the CS (auditory or visual signal)—US (shock) association in this paradigm, it would be expected that dynamic interaction with the CS-US association subserving the avoidance response would be less disrupted by scopolamine than that with the more transitory and rapidly shifting association between compartment and shock.

The understanding of anticholinergic drug effects on performance in this paradigm becomes more complete when the motor effects of these drugs are taken into account. Cholinergic drugs have well-recognized effects on the balance of the extrapyramidal motor system [see for example Janowsky et al., 1972; Wolfarth and Kolasiewicz, 1977]. Scopolamine has been reported to produce hyperactivity in the open field [Anisman et al., 1975; Wolthuis et al., 1975], thus providing another mechanism whereby active avoidance performance could be improved by scopolamine and decreased by physostigmine.

Aversively Motivated Discrimination

This categorization refers to procedures in which animals are trained to discriminate stimuli in order to avoid shock. Typically, subjects are placed in a Y or T maze with a floor that is electrified everywhere except for the arm that is illuminated. Subjects learn over several trials to approach the lit arm. Both shuttlebox avoidance and aversively motivated discriminations have been used to examine the Kamin effect, a phenomenon in which retention perform-

ance following a training session is found to first decline and then improve over the course of 10–20 days. While it is not the purpose of this revue to summarize the extensive work done in order to support or question Deutsch's [1973] theory relating cholinergic function to the Kamin effect, some generalizations on the effects of anticholinergic drugs on performance of aversively motivated discriminations can be made.

Deutsch and co-workers have shown that antimuscarinics given just before a retention test that is up to a few days after initial learning impair performance, while physostigmine or DFP (an irreversible anticholinesterase) impair performance most given before a retention test that is 5–10 days after the initial learning. Signorelli [1976] demonstrated that the effects of physostigmine lasted only as long as the drug was in the body, again indicating an effect on some dynamic component of performance rather than on memory storage. Flood et al. [1981] found that when anticholinergic drugs were centrally injected immediately after training and retention was tested a week later, performance declined. When cholinergic agonists (e.g., arecoline, oxotremorine, muscarine) or anticholinesterases were tested the same way, and baseline was adjusted to be low, some doses improved retention test performance. Deutsch and co-workers, as well as Stanes et al. [1976] have also extended Deutsch's cholinergic model to apply to the effects of physostigmine on appetitive Y-maze discrimination.

Thus, drugs that increase cholinergic function can increase or decrease performance depending on dose and retention interval; antimuscarinics either do not affect or degrade performance. Interestingly, however, when anticholinesterases disrupt performance, concurrent administration of muscarinic receptor blockers can antagonize this disruption. These data may indicate that while anticholinergic drugs usually impair acquisition or retest performance, cholinomimetics do not necessarily improve performance.

Appetitively Motivated Discrimination

Hearst [1959] provided the first report of the effects of scopolamine on go/go discriminated responding in the rat. Subjects were trained to press one of two levers for water reinforcement in the presence of one stimulus (either a clicker or a tone) and the other lever during presentations of the other stimulus. Subjects were trained to an asymptotic level of correct performance and then occasionally given scopolamine (0.2–1.0 mg/kg). Presession drug treatment resulted in a decreased percentage of correct responding (from 94.5 to 63.6) and increased the number of responses on either lever emitted when neither stimulus was present. In addition subjects developed a marked lever preference on drug sessions. Although this led to an increased number of perseverations as opposed to alternations between levers, it did not result in true perseveration: the tendency to repeat a previous response regardless of which response it is. When these subjects were given extinction sessions, scopolamine increased responses on both levers, even when no stimuli were presented or subjects were sated with water.

These data would seem to indicate that in addition to disrupting discrimination performance (perhaps through interference with the retrieval of stimulus response associations), scopolamine increases nonreinforced and extinguished responding; the overall tendency to respond is increased. However, subsequent discrimination studies have not borne this out. Milar et al. [1978] conducted a series of auditory (1,000- and 3,000-Hz tones) and visual (panel light) discrimination experiments in order to evaluate the effects of prior scopolamine (0.0625–0.5 mg/kg) treatment. Rats were trained to perform a go/no go discrimination: lever presses were reinforced with sucrose solution in the presence of one stimulus and not reinforced in the presence of another. Another stimulus (white noise) was established as a conditioned inhibitor through presentation during extinction sessions. In all experiments, excitatory and inhibitory stimulus control were similarly decreased by scopolamine. Rats given scopolamine responded more on no go trials and less on go trials.

Milar [1981] studied the effects of scopolamine (0.125–0.50 mg/kg) on a go/no go brightness discrimination in rats. Reinforcement probabilities were manipulated for both

correct responses and correct omission of responses in order to vary the degree of baseline excitatory and inhibitory stimulus control. A signal detection analysis of the data was used in order to obtain independent estimates of drug (and behavioral manipulation) effects on sensitivity (the subject's absolute ability to discriminate the stimuli) and bias (relative preference for responding or not responding, in the case of a go/no go task). Scopolamine was found to decrease sensitivity in a more difficult discrimination (less intensity difference between the discriminative stimuli) and to not affect sensitivity in a simpler intensity discrimination, in which the degree of baseline stimulus control was at ceiling. Bias was unaffected by the drug, and manipulations of reinforcement probabilities did not alter drug effects.

The latter two experiments thus do not support a disinhibitory action of antimuscarinics. Rather, they are consistent with the current encoding/retrieval hypothesis in that stimulus control of all types, which is dependent upon retrieval of previously established stimulus-response associations, is disrupted. Thus, the central discrimination processes allowing for comparison of the sample stimulus with a previously established internal representation of the rewarded (positive) discriminative stimulus may not be affected by anticholinergic drugs. Rather, cholinolytics may disrupt retrieval of the internal stimulus representation, along with its correct response associations. In this way, muscarinic receptor blockers may disrupt performance in discrimination tasks while leaving the discriminational processes intact.

Delayed Response

Two sorts of procedures exist that can provide measures of memory for recent events in animals. The first is delayed response, in which correct responding at the end of a retention interval is dependent upon retaining memory for the response-type specified before the interval. The second is delayed conditional discrimination, in which a stimulus presented at the beginning of the retention interval must be remembered and compared to the postinterval stimulus in order to determine the correct response.

Schedules of reinforcement such as fixed-interval (FI) and differential reinforcement of low rate (DRL) can be thought of as delayed response procedures. The degree of stimulus control by the schedule can be related to the degree to which responses are most efficiently partitioned over time and reinforcements received are maximized. There have been many reports of disruption of the pattern of responding on these schedules and others by atropine and scopolamine [e.g., Boren and Navarro, 1955; Hernstein, 1958]. Brown and Warburton [1971] applied signal detection analysis to the effects of scopolamine on Differential Reinforcement of Low rate (DRL)-15 sec responding by rats. Signal detection analysis revealed drug-induced changes in sensitivity but not in bias, indicating an overall reduction in control by the schedule contingencies, but not disinhibition of responding.

Several other investigators have noted that free-operant responding under simple or multiple time-based schedules is not only more accurate under "external" (presence of an exteroceptive discriminative stimulus correlated with opportunity for reinforcement) than under "internal" (no environmental cue given to signal reinforcement opportunity) control, it is also less affected by antimuscarinic agents [Laties and Weiss, 1966; Wagman and Maxey, 1969]. Ksir and Slifer [1982] have demonstrated a similar effect of scopolamine on performance of an appetitive operant discrimination. These data cannot be unambiguously interpreted, however. Degree of "internality" covaries with baseline level of response accuracy and, as has been previously discussed, anticholinergic drug effects may vary depending on degree of training and resultant level of accuracy.

The eight-arm radial maze has also been used to measure delayed response memory. Food-deprived subjects are placed on a central platform from which eight arms (walkways) radiate. Usually, a bit of food is placed at the end of each arm, and subjects are trained to travel to the arms in order to obtain all of the available food. The optimal behavior in such a situation is, therefore, to enter only arms that have not been entered before. Entering previously selected arms before all eight have been visited is considered an error. The development and

maintenance of extremely accurate win-shift behavior in rats placed in an eight-arm radial maze has been used by several researchers to study the effects of hippocampal lesions and antimuscarinics on "working memory." Eckerman et al. [1980] produced the first systematic study of drug effects on this behavior. When administered before a test session, scopolamine reduced both the accuracy and the number of arm entries. The drug did not increase response perseveration (tendency to repeat entries consecutively into the same arm). Finally, scopolamine reduced the accuracy of arm choice performance by about the same amount from the fourth choice to the eighth choice. Eckerman et al. interpreted these findings as indicative of a drug effect on discriminative control by the memorial stimuli of previous arms entered, as opposed to an effect on memory storage, which would lead to an acceleration of performance decrement over increasing retention intervals.

Stevens [1981] and Watts et al. [1981] have supported and extended these findings to show 1) scopolamine markedly reduces both acquisition and retest performance, and 2) subjects using a nonspatial strategy (i.e., a response strategy, such as always picking the arm adjacent in the clockwise direction next) were less impaired by scopolamine.

Gudding et al. [1982] confirmed the hypothesis that scopolamine did not alter memory storage in this task by slightly altering the procedure. Rats were allowed to make their first four choices and were then subjected to a 5-hr retention interval. Scopolamine injections during the interval had no effect on subsequent choice performance that was not attributable to carry-over performance effects of the drug. This was true regardless of whether subjects exhibited a spatial or nonspatial strategy.

An additional method for quantitative measurement of delayed response memory is discrete trial lever pressing. Both acquisition [Warburton, 1969] and retest performance of go/no go and go/go alternation of lever pressing is disrupted by atropine and scopolamine [Warburton and Heise, 1972; Ksir, 1974; Heise, 1975; Heise et al., 1975, 1976; Glick et al., 1979]. When variable retention intervals are used, antimuscarinics reduce accuracy following all intervals about the same amount. Since no interaction occurred between drug effects and accuracy after increasing retention intervals, Heise [1975] and others have argued that the drug does not disrupt memory storage.

Bartus and Johnson [1976], however, provided evidence that when measured against delayed response performance in rhesus monkeys, scopolamine disrupted performance more at longer retention intervals. Furthermore, physostigmine was found to antagonize scopolamine's deleterious effects [Bartus, 1978]. Since no other studies using a variable retention interval procedure have confirmed these results, it is possible that the effect might be specific to rhesus monkeys. Another possibility is that the apparent storage effect was due to a less scopolamine-induced disruption when performance (memorial stimulus control) was at ceiling, at the lowest retention intervals. Indeed, the control- and drug-induced memory functions (accuracy-by-interval plots) for delayed response performance were less obviously divergent in the later Bartus [1978] study.

Delayed Conditional Discrimination

Delayed matching-to-sample is an example of a delayed conditional discrimination. In the paired-trial form of task, subjects are usually trained to make an observing response to a stimulus on trial one, subjected to a variable retention interval, and are then presented with two stimuli on trial two, one of which is identical to that on trial one. Subjects are reinforced for a response to that "matching" stimulus.

Using this procedure with rhesus monkeys as subjects, both Bohdanecky et al. [1967] and Glick and Jarvik [1969] demonstrated that scopolamine not only decreased matching accuracy, but that it did so to the same extent at all interrial delays, including no delay at all. On no-delay trials, subjects were simultaneously presented with sample and comparison stimuli. Under these conditions, a simple discrimination was required with no dependence upon short-term memory, and yet scopolamine disrupted performance to the same degree.

Spencer [1982] trained one group of rats on a Continuous Non-Matching to sample (CNM) task, in which trials with a bright light irregularly alternated with dim light trials. Subjects were rewarded for responding when the stimulus on the current trial was different from that presented on the previous trial. Retention intervals varied from 2.5 to 10 sec. Another group of rats was trained to discriminate bright and dim lights during trials. The light intensity difference was decreased until baseline performance on trials (following the same intertrial intervals as in the CNM task) was similar to that of the rats performing the CNM task. Scopolamine injected preseasion reduced response accuracy on both discrimination and memory measures to the same extent. In addition, accuracy on no go trials was reduced by the drug to the same extent as on go trials. Accuracy in the nonmatching task under drug also declined a constant amount over retention intervals as compared to controls. These data indicate that muscarine blockade does not affect memory storage in these tasks and produces a deficit in tasks depending on memory for both recent stimulus occurrences (CNM procedure) and constant stimulus–response–reinforcement associations (both CNM and discrimination procedures).

CONCLUSIONS

Based on the data presented above, the following generalizations on the effects of antimuscarinic drugs in animals seem appropriate. First, behavioral effects of the type discussed in this chapter are the result of drug action within the central nervous sytem. Second, the processes of habituation and behavioral inhibition are not selectively affected by antimuscarinics. Third, the types of associational link (operant or classical), task motivation, and memory requirements do not seem to modify the pattern of behavioral effects produced by antimuscarinics. Fourth, memory storage itself is rarely affected by muscarinic blockers.

It has been proposed that all of the experimental data reviewed above are best accounted for by the hypothesis that anticholinergics alter learning and memory performance by interfering with or distorting the input (encoding)/output (retrieval) processes involved in dynamic interactions with memory storage. This is not to say that other hypotheses might not account more parsimoniously for certain portions of the reviewed data. Rather, the current hypothesis is presented as the simplest theoretical construct that is at least consonant with the results from all of the various behavioral procedures.

A further proposition is that the strength or situational generality of the associative link affects the probability of its successful retrieval during or following exposure to antimuscarinic drugs. This notion was prompted by consideration of the spontaneous alternation data, which strongly indicate that increasing the differential stimulus salience in T-maze arms or increasing the degree of exposure to stimuli accompanying an arm choice decreases the effect of antimuscarinic drugs. Future research on cholinolytic drug effects might therefore profit by devoting additional study to the interaction between the number of exposures to conditional associations, the diversity of situations or stimuli accompanying such exposures, graded variations in degree of stimulus control in a particular task, and resulting anticholinergic drug effects.

The hypothesis that encoding/retrieval is the locus of antimuscarinic drug effects is consistent with the notion that the development of a cholinergic lesion during aging or the onset of a dementic state results in impaired learning and memory. Thus, it may be valid to model dementia in animals by inducing a functional cholinergic lesion through the use of antimuscarinic drugs. The development of geriatric and antidementic drugs could therefore be aided by testing potential drugs for their ability to antagonize cholinolytic drug effects in certain, well-selected behavioral tasks. Throughout this review, an attempt has been made to show how several hypotheses of the locus of behavioral drug effect can apply to performance in most tasks. Therefore, if one is to model the cognitive deficits of aging and dementia in drug-treated animals correctly, it is imperative to choose a behavioral task in which drug

effects on performance (as well as antagonism of those effects) can reliably and objectively be partitioned. That is, it must be possible to separate drug effects on motivation and sensorimotor capacity from those on learning or memory. The most promising current approach in this direction is thus the comparison of drug effects in a multiple intertrial interval delayed response (or delayed conditional discrimination) procedure with those on a task with a lower memory load, such as discrimination (e.g., Spencer, 1982).

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MK-801 Impairs Recognition Memory in Rhesus Monkeys: Comparison with Cholinergic Drugs[1]

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ABSTRACT

Both N-methyl-D-aspartate (NMDA) and cholinergic receptors are thought to participate in processes of learning and memory. The effects of the noncompetitive NMDA antagonist ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine) MK-801 on recognition memory in rhesus monkeys performing a computer-automated version of delayed nonmatching-to-sample DNMS were compared to those of the cholinergic compounds physostigmine and scopolamine. In the sample phase of the test, 20 symbols were presented sequentially every 30 sec on a color monitor fitted with a touch-sensitive screen. These symbols were then presented again in the same order as before, but each symbol was now paired with a different novel symbol. A monkey was rewarded with a food pellet if it touched the symbol in the sample phase and the previously unseen symbol in the choic phase. Physostigmine (3.2, 10 and 32 μg/kg), scopolamine (3.2 10, 17.8 and 32 μg/kg) or MK-801 (3.2, 10 and 32 μg/kg) wa injected i.m. 20, 20 and 30 min before testing, respectively. Th highest doses of both MK-801 and scopolamine significantl impaired performance. In addition, scopolamine, but not MK 801, prolonged response latency, whereas MK-801, but nc scopolamine, increased response bias. Physostigmine produce a small but significant increase in correct responses at th intermediate dose, but not at the highest dose. These result suggest that both the glutamatergic and the cholinergic system participate in visual recognition memory in monkeys, thoug probably by different mechanisms.

Excitatory amino acid receptors are now thought to mediate major excitatory synaptic transmission in the mammalian central nervous system (Cotman and Iversen, 1987). Among the multiple classes of excitatory amino acid receptors, the NMDA receptor is the best characterized one in terms of specific agonists and antagonists. Blockade of this receptor, which has an especially high density in the hippocampus, prevents the development of long-term potentiation, a mechanism of synaptic modification, in this same structure (Collingridge *et al.*, 1983; Collingridge and Bliss, 1987). These results have directed attention to the role of the NMDA receptor in processes of learning and memory.

MK-801 (Dizocilpine) is a potent noncompetitive NMDA antagonist that shows a high affinity for the phencyclidine binding site, which is thought to regulate the receptor-ionophore complex of the NMDA receptor (Wong *et al.*, 1986). Although MK-801 has been shown to impair some forms of learning in rodents (Parada-Turska and Turski, 1990; Pontecorvo *et al.*, 1991; Shapiro and Caramanos, 1990), there ar presently no data available on the mnemonic effects of thi compound in nonhuman primates.

Whereas a definitive role for the glutamate receptor in proc esses of learning and memory remains to be shown, there is considerable body of data indicating such a role for the mus carinic-type of cholinergic receptor. Scopolamine, a muscarinic receptor antagonist, has been shown in numerous studies t impair learning and memory under a variety of testing condi tions not only in small animals (*e.g.*, Bohdanecky and Jarvik 1967; Deutsch, 1971; Spencer and Lal, 1973), but also in mon keys (Aigner and Mishkin, 1986; Aigner *et al.*, 1991a,b; Rupniak *et al.*, 1991) and in humans (Crow and Grove-White, 1973 Drachman and Leavitt, 1974; Petersen, 1977; Ghoneim and Mewaldt, 1977). Conversely, some cholinesterase inhibitors have been shown to enhance learning and memory under some conditions in both animals (Aigner and Mishkin, 1986; Ridley *et al.*, 1987) and humans (Davis *et al.*, 1978; Christie *et al.* 1981).

We previously reported (Aigner and Mishkin, 1986) that physostigmine and scopolamine produced dose-related increases and decreases, respectively, in the number of objects correctly remembered by rhesus monkeys performing a DNMS

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ABBREVIATIONS: NMDA, N-methyl-D-aspartate; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine; DNMS, delayed nonmatching-to-sample; WGTA, Wisconsin General Testing Apparatus.

task with trial-unique objects presented in a Wisconsin General Testing Apparatus (WGTA). In the present study, we used a computer-automated version of the DNMS task and compared the effects of the NMDA antagonist MK-801 with those of the cholinergic drugs, scopolamine and physostigmine.

Materials and Methods

Subjects. This study was conducted under a protocol approved by the National Institute of Mental Health Animal Care and Use Committee and was in accordance with The Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health. Four male rhesus monkeys (*Macaca mulatta*) weighing 6 to 8.5 kg at the beginning of testing were used. All animals had prior behavioral testing experience, and all had previously received acute injections of low doses of cholinergic drugs, although none had been tested during the 2 months before the present investigation. Food intake was restricted to the amount earned during each daily session plus a regular afternoon feeding of monkey chow (Purina, St. Louis, MO) at least 2 hr after testing was completed. Water was freely available in the home cage.

Apparatus. All testing was conducted in a darkened, sound-attenuated cubicle (120 cm × 60 cm × 100 cm). A color monitor fitted with a touch-sensitive screen (Microtouch, Wilmington, MA) was located on a shelf on one wall of the cubicle. The monkey sat comfortably in a restraint chair that allowed free movement of arms and hands, directly in front of and within easy reach of the monitor. A 300-mg banana-flavored pellet (Noyes Co., Lancaster, NJ) could be delivered from a food dispenser located outside the cubicle to a receptacle in front of the animal.

Behavioral procedure. The task used was a computer-automated version of DNMS with a list length of 20 trial-unique graphic symbols. Each of the symbols occupied an area of approximately 50 cm^2 on screen. The symbols for each session were drawn from a supply of 1200 different symbols (combinations of 69 ASCII characters, seven colors and four angles of rotation) that had been randomly divided into 60 sets of 20 symbols each. The sets were used in sequence, four sets per day, until all of the sets had been presented once, after which the process was repeated as often as necessary to complete the testing. An animal was thus shown a particular symbol no more often than once every 3 weeks. These parameters were chosen to be as similar as possible to the method used previously in the WGTA (Aigner and Mishkin, 1986).

In the sample phase of the task, each symbol in the set of 20 was displayed in the center of the color monitor. The animal obtained a banana pellet by touching each of these symbols. Then, after all 20 had been presented, each sample symbol was paired with a novel symbol. In this choice phase the animal was rewarded with a pellet if it touched the previously unseen symbol. The familiar and novel symbols were presented on the left and right portions of the screen approximately 10 cm apart, and the positions of the two varied in a pseudorandom manner. The time between symbol presentations in both the sample and choice phases was 30 sec. An interval of 10 min thus elapsed from the time a given symbol in the list was first shown as the sample until it was paired with a novel symbol in the choice phase. Two lists of 20 symbols each, for a total of 40 trials, comprised a daily session. Testing was conducted 5 days a week. When day-to-day performance was stable for all of the monkeys, drug testing was begun. Percent correct, response latency and response bias (Index Y) measures were automatically calculated at the end of the session. Index Y, which is a measure of response bias developed for memory studies such as the present one (Sahgal, 1987), equals the absolute difference between the two (right and left) alternative hit frequencies, divided by the sum of the frequencies.

Drug testing. Dose-effect functions were first determined for physostigmine (3.2, 10 and 32 μg/kg), followed by scopolamine (3.2, 10, 17.8 and 32 μg/kg) and then for MK-801 (3.2, 10 and 32 μg/kg). All doses refer to the salt forms of the drugs: physostigmine salicylate (Antilirium, Forest Pharmaceuticals Inc., St. Louis, MO), scopolamine hydrobromide (Sigma Chemical Company, St. Louis, MO), and MK-801 hydrochloride (Research Biochemicals Incorporated, Natick, MA). All compounds were dissolved in distilled water for intramuscular injection. The injection volume was kept constant at 0.1 ml/kg irrespective of dose. Each series of doses was tested twice, first in an ascending order and then in a descending order. Control injections of saline were also given before each series of drugs and after completion of all drug testing. Drugs were administered no more often than twice per week. At least two noninjection control sessions intervened between sessions in which either a drug or saline was administered. All injections were made while the animals were seated in the primate chair and were administered 20, 20 and 30 min before the start of the session for physostigmine, scopolamine and MK-801, respectively.

Statistical analysis. Percent correct data were analyzed by Friedman's nonparametric test, followed by Wilcoxon matched-pairs test. The data for response latency and Index Y were tested with a two-way analysis of variance with repeated measures followed by posthoc, two-tailed, paired *t* tests.

Results

MK-801. The effects of MK-801 on visual recognition memory in the four monkeys are shown in figure 1. MK-801 caused a significant decrease in the percentage of correct choices ($\chi^2 = 10.39$; $P < .02$). The highest dose of MK-801 produced a significant decrease in percent correct responses ($P = .01$) and also significantly raised Index Y ($F = 5.34$; $df = 3,16$; $P < .01$) at the highest dose ($P < .05$). By contrast, MK-801 had no effect on response latency in either the sample or choice phases. After the 32 μg/kg dose of MK-801, slight motor incoordination was observed for approximately 2 hr in some of the monkeys after they returned to their home cages.

Scopolamine. The effects of scopolamine on performance of the automated DNMS task are shown in figure 2. Scopolamine caused a dose-dependent impairment ($\chi^2 = 14.8$; $P < .01$), with the highest dose of scopolamine producing a significant impairment in percent correct ($P = .05$). After the 32 μg/kg dose, one monkey performed at only 54.2% correct percent on the first list of 20 symbols and then did not respond at all on the second list. This animal did not complete testing and so these data were omitted from the statistical analysis. At the highest doses, signs of peripheral cholinergic antagonism, such as dry mouth (some animals did not consume the banana pellets they earned) and pupillary dilation, were observed. Scopolamine significantly increased response latency in both sample ($F = 21.78$; $df = 2,15$; $P < .001$) and choice phase ($F = 4.64$; $df = 2,15$; $P < .025$). In the sample phase, there were significant

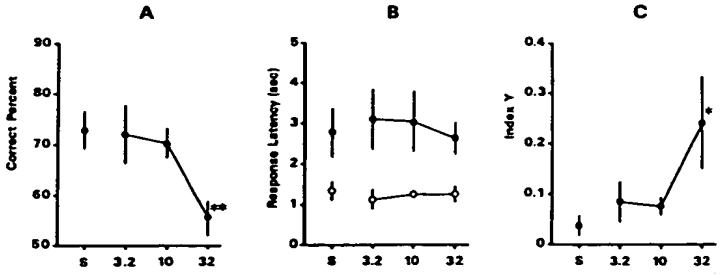

Fig. 1. Effect of MK-801 on delayed nonmatching-to-sample in rhesus monkeys. Scores are group means (±S.E.M.) as a function of dose (μg/kg). A, percent correct, B, response latency: filled circles, choice phase; open circles, sample phase. C, Index Y. Monkeys received saline or the indicated dose of MK-801 i.m. 30 min befor t sting. *,**$P < .05, .01$ *vs.* saline treatment, respectively.

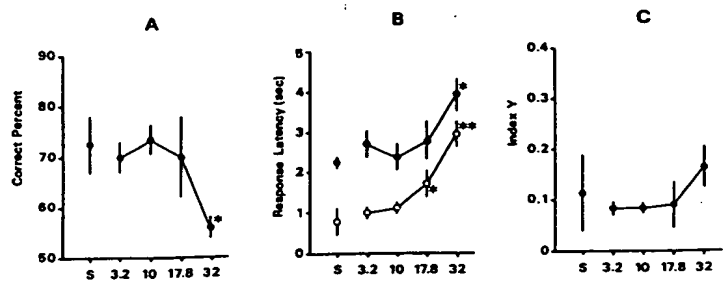

Fig. 2. Effect of scopolamine on delayed nonmatching-to-sample in rhesus monkeys. Scores are group means (±S.E.M.) as a function of dose (μg/kg). A, percent correct; B, response latency: filled circles, choice phase; open circles, sample phase. C, Index Y. Monkeys received saline or th indicated dose of scopolamine i.m. 20 min before testing. *,**P < .05, .01 *vs.* saline treatment, respectively.

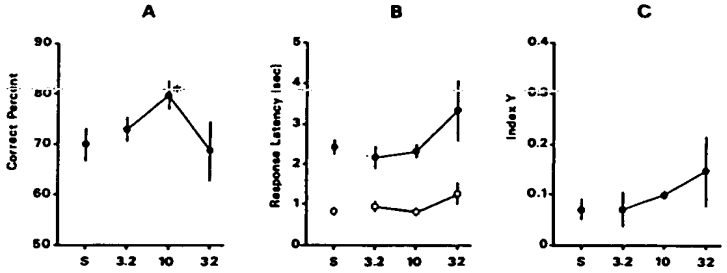

Fig. 3. Effect of physostigmine on delayed nonmatching-to-sample in rhesus monkeys. Scores are group means (±S.E.M.) as a function of dose (μg/kg). A, percent correct; B, response latency: filled circles, choice phase; open circles, sample phase. C, Index Y. Monkeys received saline or th indicated dose of physostigmine i.m. 20 min before testing. *P < .05 *vs.* saline treatment.

increases in response latencies at the two higher doses. In the choice phase, there was a significant increase in response latency only at the highest dose (32 μg/kg). Index Y was not affected by scopolamine administration.

Physostigmine. The dose-effect functions for physostigmine are shown in figure 3. A significant improvement was obtained following physostigmine administration ($\chi^2 = 8.888$; $P < .05$). The 10 μg/kg dose of physostigmine produced a significant increase ($P < .05$) in percent correct choices (79.7%) compared with saline-treated control (69.8%). Group analysis showed no difference between the saline and 32 μg/kg treatment, although one of the four monkeys performed more poorly after the highest dose than after the saline control. Response latency was not significantly affected in either the sample or choice phases. Also, physostigmine had no effect on Index Y.

Discussion

The results of this study extend the findings from other laboratories that the noncompetitive NMDA antagonist MK-801 impairs mnemonic performance in animals, in this case in primates performing an automated visual-recognition memory task. Previous studies in rodents showed that MK-801, as well as other NMDA antagonists, impairs performance in a variety of paradigms including spatial memory tasks such as the water maze (Whishaw and Auer, 1989; Robinson *et al.*, 1989; Heale and Harley, 1990), the radial-arm maze (Shapiro and Caramanos, 1990; Ward *et al.*, 1990; Wozniak *et al.*, 1990; Butelman, 1990; Bischoff and Tiedtke, 1992), aversively motivated avoidance (Venable and Kelly, 1990; Spangler *et al.*, 1991) and delayed response (Tan *et al.*, 1989; Pontecorvo *et al.*, 1991).

Morris *et al.* (1986) attributed the mechanism for the impairment induced by an NMDA antagonist on spatial memory to the inhibition of long-term potentiation in the hippocampus. Robinson *et al.* (1989) further suggested that the NMDA antagonist-induced impairment mimicked the effects of hippocampal lesions. In nonhuman primates, performance on the WGTA/DNMS task with trial-unique objects is severely disrupted by large medial temporal lesions that include the amygdala, the hippocampus and underlying cortex (Mishkin, 1978). It is tempting to suggest that the site of action for the impairment observed in the present study is also the hippocampus, where the density of NMDA receptors is thought to be the highest in the brain (Monaghan and Cotman, 1986). However, analysis of the response characteristics showed that at doses that impaired task performance, MK-801 increased response bias. That is to say, the impaired performance was associated with the animals' tendencies to limit their responses to the symbols on either the left or right side of the screen during the choice phase. The brain area through which MK-801 might have affected response bias (Index Y) can only be speculated about at this time, although there is evidence that the inferior prefrontal cortex may be a candidate site for this behavioral effect. It has been shown previously that aspiration lesions of this cortical region in monkeys, like MK-801, produce an increase in side preference in the WGTA/DNMS task (Kowalska *et al.*, 1991). Further studies in both normal and lesioned monkeys may help to delineate the site and mechanism of this action of MK-801.

Scopolamine has been shown previously to impair performance of primates in other memory testing procedures (Aigner and Mishkin, 1986; Aigner *et al.*, 1991a,b; Rupniak *et al.*, 1991). The present results extend these findings to show that scopolamine also impairs performance in an automated memory testing task. The possible contribution of attentional, perceptual and motivational effects of scopolamine to the impairment cannot be ruled out completely. Because muscarinic receptors are found in both peripheral and central sites, such effects of scopolamine could be due to its action in any number of areas. Scopolamine is known to disrupt performance on some types of visual and auditory discrimination tasks (Evans, 1975; van Haaren and van Hest, 1989), as well as on attentional tasks (Wesnes and Warburton, 1984). It is interesting, however, that scopolamine did not affect response bias, even at the highest dose that significantly decreased correct choices. This suggests that monkeys were still comparing the symbols on the choice test, even after the highest dose of scopolamine. The mechanisms by which response times were prolonged by scopolamine are unknown, although it is likely that peripheral actions of the drug may be responsible. Under some testing conditions, administration of the peripheral cholinesterase inhibitor neostigmine can reduce the incidence of the side effects such as dry mouth and dilated pupils (T. G. Aigner, unpublished observations). Similar studies are therefore needed to test the effects of combining neostigmine and scopolamine on reaction time. It is also possible, however, that as the dose of scopolamine is increased, this drug may be acting directly on central nervous system motor or extrapyramidal pathways involved in the initiation and regulation of movement.

Numerous clinical studies have examined the possible memory-enhancing effects of cholinomimetics in normal volunteers as well as patients with Alzheimer's disease (Brinkman and Gershon, 1983). The results have been equivocal so far, al-

though some studies have reported that cholinesterase inhibitors such as physostigmine and tetrahydroaminoacridine alleviate the cognitive impairment in this disease (Davis *et al.*, 1978; Davis and Mohs, 1982; Muramoto *et al.*, 1979; Summers *et al.*, 1986). Results from studies in animals have suggested that cholinesterase inhibitors can indeed improve memory. Physostigmine has been reported to have such an effect under a variety of test conditions and in a variety of experimental preparations, *e.g.*, normal monkeys (Aigner and Mishkin, 1986), scopolamine-treated monkeys (Rupniak *et al.*, 1989) and rats with nucleus basalis magnocellularis lesions (Murray and Fibiger, 1985; Mandel and Thal, 1988). In the present study, physostigmine improved visual recognition memory performance, the dose-response relationship exhibiting an inverted U shape, although the effect was mild and significant improvement was limited to a single dose. These results correspond well with clinical studies indicating that the therapeutic range for physostigmine is narrow and that it is necessary to individualize doses (Mohs *et al.*, 1985; Schwartz and Kohlstaedt, 1986). The inverted U-shaped dose-response curve has also been reported previously in animal studies (Flood *et al.*, 1981; Aigner and Mishkin, 1986). It is worth noting that the dose of physostigmine that improved performance in the present study had no effect on response latency. It appears therefore that physostigmine did not affect attentional or motivational processes, but selectively improved memory at the 10 μg/kg dose.

The results on the effects of cholinergic drugs reported here are similar to those obtained previously on the DNMS task with trial-unique objects in monkeys (Aigner and Mishkin, 1986). However, although both improvements with physostigmine and impairments with scopolamine were reported in the earlier study, the dose levels that produced the effects were lower than those used here. The automated-testing procedure seems therefore to be somewhat less sensitive than the one used in the WGTA. This could be due to several factors. The graphic symbols were two dimensional and each was in one of only seven colors. Therefore, the discriminability of the symbols was less than that of the multicolored, three-dimensional junk objects. Studies are now in progress to examine how more discriminable two-dimensional visual stimuli interact with the effects of cholinergic compounds. Obviously, however, the automated-testing procedure offers certain advantages over the WGTA procedures, *e.g.*, unintentional experimenter bias is eliminated, reaction times can be accurately measured and intertrial intervals can be precisely controlled.

In conclusion, we compared the effects of the NMDA antagonist MK-801 with those of the cholinergic drugs scopolamine and physostigmine on a computer-automated recognition memory task in rhesus monkeys. Both MK-801 and scopolamine impaired recognition memory, although probably by different mechanisms, as MK-801, but not scopolamine, increased response bias, and scopolamine, but not MK-801, increased reaction time. Physostigmine, by contrast, produced a moderate, but significant recognition memory improvement without affecting either response measure. These results in primates provide further evidence implicating the NMDA and cholinergic rec ptors in processes of memory, and encourage investigations to identify the cerebral locus as well as the mechanisms of their action.

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Comparison of the effects of four cholinomimetic agents on cognition in primates following disruption by scopolamine or by lists of objects

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Abstract. The ability of four central cholinomimetics to reverse a scopolamine-induced spatial memory impairment or to improve visual recognition memory in primates was examined. Physostigmine (0.04–0.08 mg/kg IM) fully reversed the effects of scopolamine (0.03 mg/kg). Coadministration of pilocarpine (3.0–5.0 mg/kg) caused partial reversal of the scopolamine impairment after intermediate or long retention intervals (10 or 20 s). Treatment with arecoline (0.1–1.8 mg/kg) or nicotine (1.0–2.0 mg/kg) generally did not reverse the effects of scopolamine. A task in which memory could be taxed by increasing the number of visual stimuli presented appeared more sensitive to the effects of cholinomimetics on cognition than the scopolamine reversal model. In this paradigm treatment with physostigmine (0.001, 0.01 or 0.03 mg/kg) increased choice accuracy from about 55 to 70% correct. Arecoline improved performance at one dose only (0.1 mg/kg) which also induced marked adverse side-effects (salivation and tremor). Pilocarpine improved performance in the dose range 0.125–0.35 mg/kg, but not at higher doses which also induced marked salivation. Treatment with nicotine (0.001–2.0 mg/kg tended to improve performance but this did not reach statistical significance. The relevance of these findings for studies in man and for animal models of dementia is discussed.

Key words: Delayed nonmatching-to-sample – Spatial delayed response – Cholinomimetics – Scopolamine – Rhesus monkey

Cognitive improvements in Alzheimer's disease have most reliably been obtained following treatment with cholinomimetic agents such as physostigmine (Christie et al. 1981; Davis et al. 1981), arecoline (Christie et al. 1981) and nicotine (Newhouse et al. 1988). Unfortunately despite the apparent efficacy of these agents, their clinical utility is compromised by their short duration of action, narrow therapeutic window and unpleasant side-effects. Although there is a correlation between cholinergic dysfunction and the degree of cognitive decline in Alzheimer's disease (Perry et al. 1978), the contribution of numerous other neurotransmitter pathways which are also affected by the disease (Rossor and Iversen 1986) has not yet been fully assessed.

Accurate preclinical screening for more effective and acceptable treatments requires animal models which are able to detect the effects of drugs on cognitive processes resembling those affected in dementia, and which reflect the known clinical pharmacology of the disease. Unlike other species, primates are able to perform many complex behavioural tasks identical to those impaired in human amnesic states, including dementia (Freedman and Oscar-Berman 1986; Moss et al. 1986; Flicker et al. 1987; Irle et al. 1988; Sahakian et al. 1988).

Two examples of such primate tasks require the animal to retain information relating to a specific physical attribute of a sample stimulus (such as its visual characteristics or spatial location). Choice accuracy may then be disrupted following a retention interval either with a pharmacological agent such as scopolamine (Bartus and Johnson 1976) or by presenting a serial list of intervening stimuli which are also required to be remembered (Aigner and Mishkin 1986). Administration of the acetylcholinesterase inhibitor physostigmine is able to improve performance in both paradigms (Bartus 1978; Aigner and Mishkin 1986). However, at present it is not known whether the nature of the cognitive impairment induced by these two different manipulations is similar, or whether they are equally sensitive to the effects other, directly acting, cholinomimetic agents which improve cognition in man. In the present paper we have directly compared the ability of four central cholinergic agonists (physostigmine, arecoline, pilocarpine and nicotine) to improve cognitive performance in primates either following disruption by scopolamine or by lists of intervening stimuli.

Materials and methods

Subjects

The subjects were 17 adolescent male rhesus monkeys (*Macaca mulatta*; 3.0–4.5 kg). Of these, 13 were trained in the spatial delayed response task and 4 in the visual recognition test. Monkeys were housed with others in pairs and maintained on the recommended daily dietary allowance of Mazurri powder prepared as mash (Special Diet Services, Essex), supplemented with fresh fruit or vegetables, which they received after the completion of cognitive testing each day.

Spatial delayed response task

Apparatus. Testing was carried out using an automated apparatus based on a design originally developed by D. Gaf-

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fan, Oxford. The monkey sat alone in a sound-attenuated cubicle in a transport cage through which he could reach a touch-sensitive 20″ colour television screen equipped with infra-red photocell emitters and receivers (Mellordata Ltd, Suffolk). On the screen were displayed visual stimuli generated via a Pluto II graphics board (IO Research Ltd, London) controlled by an IBMpcXT microcomputer (IBM, Essex). The delivery of food rewards was contingent upon the monkey touching the appropriate stimulus as described below. Rewards were delivered to a central food hopper located infront of the transport cage and below the television screen. The animal could be observed remotely by means of a camera positioned above the transport cage.

Training. The task was designed to replicate as closely as possible the method described by Bartus and Johnson (1976). During initial training a single white square (approximately 3 × 3 cm) was displayed on the screen at the beginning of a trial in one of nine possible spatial locations available in a 3 × 3 matrix. The position of this acquisition stimulus was varied randomly from trial to trial according to a unique sequence each day. The stimulus remained on display until it was touched by the monkey to initiate the trial. The stimulus then immediately disappeared and the screen remained blank throughout a 2 s delay, at the end of which nine choice stimuli (white squares identical to the acquisition stimulus) located in each of the available spatial locations were presented simultaneously on the screen. The monkey was required to touch the square occupying the same spatial position as the acquisition stimulus in order to obtain a reward (peanut, banana pellet or similar titbit). An incorrect choice was signalled by a brief train of clicks, and that trial was then repeated until the correct response was emitted. If the animal failed to make his choice within 5 s, the screen was blanked and that trial was repeated from the beginning after a 2 s intertrial interval. A session consisted of 90 such trials.

Once an animal had reached a criterion of 90% correct on 2 consecutive days he progressed to the next stage of training in which trials with longer retention intervals were gradually introduced, 10 trials at a time, keeping the total number of trials constant at 90. No correction procedure was used during this stage. An animal would typically proceed in this way initially from 90 trials with a 2 s retention interval to (i) 60 trials at 2 s and 30 trials at 5 s, then (ii) 30 trials at 2 s, 30 trials at 5 s and 30 trials at 10 s, and finally (iii) 30 trials each at 2, 10 and 20 s delays. Animals were allowed to advance to the next stage of training only if their performance at the 2 s delay was maintained at 90% correct or better. The order in which retention intervals of different duration were presented was randomised daily. If a monkey attempted to bridge the delay simply by keeping his hand on the screen throughout the retention interval, the trial was aborted and restarted following a 10 s delay. Each session lasted approximately 30 min.

Visual recognition memory task

Apparatus. The monkey sat facing the experimenter in a Wisconsin General Test Apparatus, separated from him by two moving partitions (a one-way viewing panel on the experimenter's side and an opaque screen on the monkey's side). Between the partitions was a test tray (20 × 90 cm) with three foodwells, one in the centre and two spaced equidistantly (15 cm) on either side. These could be baited with a food reward by the experimenter and covered with a three-dimensional junk object which the monkey had to displace in order to retrieve the reward. The junk objects (toys, empty containers and ornaments) served as visibly distinct stimuli drawn from a sub-grouped pool of over 1200. Any given object would be presented approximately once every 2–3 weeks.

Training. The task was a modification of the nonmatching-to-sample procedure described by Aigner and Mishkin (1986) such that at the retention test the monkeys chose between three, rather than two, junk objects, thus reducing chance performance from 50 to 33% correct. During the initial stage of training, at the start of a trial the experimenter baited one of the foodwells according to a random sequence, covered it with a junk object and raised the opaque partition. After the monkey had displaced the object and taken his reward, the screen was again lowered, the junk object removed and another foodwell was baited and covered with a second object. The screen was raised after approximately 15 s, and lowered once the monkey had taken his reward. The choice test then followed. Again, one foodwell was selected at random to be baited and covered with a third, novel object. The objects already seen by the monkey were selected in the same serial order, and placed over the two remaining unbaited foodwells. In order to obtain the reward, the animal had to recognise the familiar objects, avoid them and correctly identify the novel or "non-matching" object. A session consisted of 30 such trials. Once the animals had reached a criterion of 90% correct on this task, the number of sample objects was gradually increased in stages to form an acquisition list of 4, 6, 10, 20, 30 or 60 stimuli, titrated for each individual animal until baseline performance remained stable at around 60% correct. At this stage each session consisted of 30 trials composed of either one list of 60 acquisition stimuli (two animals), or two lists of 30 (two animals), and lasted around 40 min. The task may be illustrated schematically as follows: an acquisition list of up to 60 objects, each rewarded (A^+, B^+, C^+, D^+ ... R^+) followed by the retention test of up to 30 consecutive trials (trial 1 = B^- S^+ A^-; trial 2 = T^+ C^- D^-).

Drug administration. Drugs were freshly prepared in 0.9% saline using an injection volume of 0.1 ml/kg IM. For the spatial memory task all test drugs were co-administered with 0.03 mg/kg scopolamine hydrobromide 30 min before behavioural testing. This dose of scopolamine was selected on the basis of previous studies as the lowest dose consistently producing a greater disruption of choice accuracy after long than short retention intervals. Physostigmine salicylate was examined at 8 doses in the range 0.01–0.08 mg/kg, arecoline hydrochloride at 12 doses in the range 0.1–1.8 mg/kg, pilocarpine nitrate at 6 doses in the range of 2.0–5.0 mg/kg, and nicotine hydrogen tartrate at 9 doses in the range 0.001–2.0 mg/kg. With the exception of physostigmine, all animals received every treatment. For the visual recognition memory task animals received a 20 min pretreatment with either saline, physostigmine salicylate (six doses in the range 0.0003–0.06 mg/kg), arecoline hydrochloride (three doses in the range 0.05–0.1 mg/kg) or pilocarpine nitrate (four doses in the range 0.25–1.0 mg/kg). Nicotine hydrogen tartrate was administered at eight doses in the

range 0.001–2.0 mg/kg 10 min prior to testing. All doses are expressed as the respective salt. Compounds were obtained from Sigma Chemical Co. Ltd, Dorset. Initial dose ranges for investigation were based on those used by Bartus (1978) and Aigner and Mishkin (1986) for physostigmine, Ridley et al. (1987) for arecoline and pilocarpine, and Elrod et al. (1988) for nicotine. For any given treatment, doses were administered in a random order where possible such that not more than three animals received the same treatment on any day. At least one drug-free day elapsed between treatments.

Statistical analysis. Data were subjected to one-way analysis of variance with repeated measures where appropriate followed by comparisons with control or scopolamine-induced performance using one-tailed Dunnett's tests.

Results

Spatial delayed-response task

Effect of scopolamine. On days when monkeys received no drug treatment, response accuracy was maintained in the region of 90–100% correct regardless of the length of the retention interval (2, 10 or 20 s; Fig. 1 A–D). Administration of the antimuscarinic agent scopolamine hydrobromide (0.03 mg/kg IM, 30 min previously) typically induced a marked disruption of choice accuracy after long (10 and 20 s) but not short (2 s) retention intervals. After the longest delay performance was reduced close to chance levels (20–30% correct; chance = 11%, Fig. 1). Session length was not affected by scopolamine or any other drug treatment (around 30 min), indicating that ability to perform the task was not impeded.

Effect of cholinomimetic agents on scopolamine-induced impairment

Physostigmine. Physostigmine salicylate was able to reverse the effects of scopolamine on spatial memory dose-dependently. Treatment with the highest dose of physostigmine (0.08 mg/kg) improved performance by comparison with scopolamine alone even at the shortest retention interval (2 s; $F=3.11$, $P=0.01$). Similarly, following coadministration of physostigmine in the dose range 0.04–0.08 mg/kg, the effects of scopolamine after retention intervals of 10 or 20 s were fully reversed (that is, the number of correct responses was significantly greater than after treatment with scopolamine alone, and lay in the same range of performance observed in the undrugged state ($F=8.70$, $P<0.001$ at 10 s; $F=12.16$, $P<0.001$ at 20 s; Fig. 1 *A*). Treatment with a lower dose (0.03 mg/kg) also fully reversed the effects of scopolamine after 10, but not 20 s retention intervals. Lower doses of physostigmine (0.01 and 0.02 mg/kg) did not reverse the effects of scopolamine at any retention interval (Fig. 1 *A*). Physostigmine was well tolerated by all subjects, even at the highest doses. No adverse signs were observed.

Arecoline. Coadministration of arecoline hydrochloride (0.1–1.8 mg/kg IM) with scopolamine failed to improve choice accuracy by comparison with the effects of scopolamine alone at any retention interval ($F=1.87$, $P=0.056$ at 2 s; $F=1.28$, $P=0.26$ at 10 s, and $F=1.17$, $P=0.33$ at 20 s; Fig. 1 *B*). Higher doses were not examined owing to the induction of adverse signs by 1.8 mg/kg arecoline. These included flushing or pallor, tremor and incoordination and were most prominent during the first 5–10 min after drug administration.

Pilocarpine. Treatment with pilocarpine nitrate (2.0–5.0 mg/kg IM) caused a dose-dependent partial reversal of the effects of scopolamine after intermediate (10 s) and long (20 s) retention intervals (that is, performance was significantly different from that observed both in undrugged animals *and* in scopolamine-treated monkeys: $F=9.05$, $P<0.001$ at 10 s and $F=8.70$, $P<0.001$ at 20 s; Fig. 1 *C*). At a dose of 4.0 mg/kg or higher, adverse signs were apparent which included pallor, salivation, retching and emesis. These signs began to subside after the completion of behavioural testing.

Nicotine. Coadministration of nicotine hydrogen tartrate (0.001–2.0 mg/kg) with scopolamine did not affect response accuracy after retention intervals of 10 or 20 s by comparison with scopolamine treatment alone ($F=0.84$, $P=0.58$ and $F=0.63$, $P=0.76$, respectively; Fig. 1 *D*). Unusually, however, scopolamine also impaired performance after a 2 s delay in this experiment, and performance remained below undrugged levels after coadministration of 1–5 μg/kg nicotine ($F=3.44$, $P=0.003$). With higher doses of nicotine (up to 2 mg/kg), scores were similar to those in undrugged animals, and at 1 mg/kg were significantly better than after treatment with scopolamine alone ($F=2.82$, $P=0.01$). No adverse signs were observed at any dose of nicotine examined.

Visual recognition memory task

Physostigmine. Treatment with physostigmine salicylate (0.001, 0.01 or 0.03 mg/kg) increased choice accuracy from approximately 55% in the undrugged state to about 70% correct ($F=7.78$, $P<0.001$). An intermediate dose (0.003 mg/kg) also tended to improve performance, but this effect failed to reach significance. Treatment with saline, or a low dose of physostigmine (0.0003 mg/kg) had no effect on performance (Fig. 2 *A*). Two monkeys refused to be tested after treatment with the highest dose (0.06 mg/kg). Of the remaining animals, one showed a modest improvement from baseline (67% correct) and the other an impairment (40% correct). No overt adverse signs were observed at any dose examined.

Arecoline. Administration of arecoline hydrochloride caused a steep increase in choice accuracy at the highest dose (0.1 mg/kg) but not at lower doses (0.05 and 0.075 mg/kg; $F=3.38$, $P=0.039$; Fig. 2 *B*). Although the performance of three of the four animals was improved by 0.1 mg/kg arecoline, this was accompanied by pallor, emesis and salivation; the fourth animal also exhibited tremor and mild ataxia and refused to test. This animal did not appear to benefit from treatment with arecoline at any dose examined.

Pilocarpine. The dose-response curve for recognition memory following treatment with pilocarpine was bell-shaped (Fig. 2 *C*). There was an increase in choice accuracy from around 57–65% correct in the dose range 0.125–0.35 mg/kg

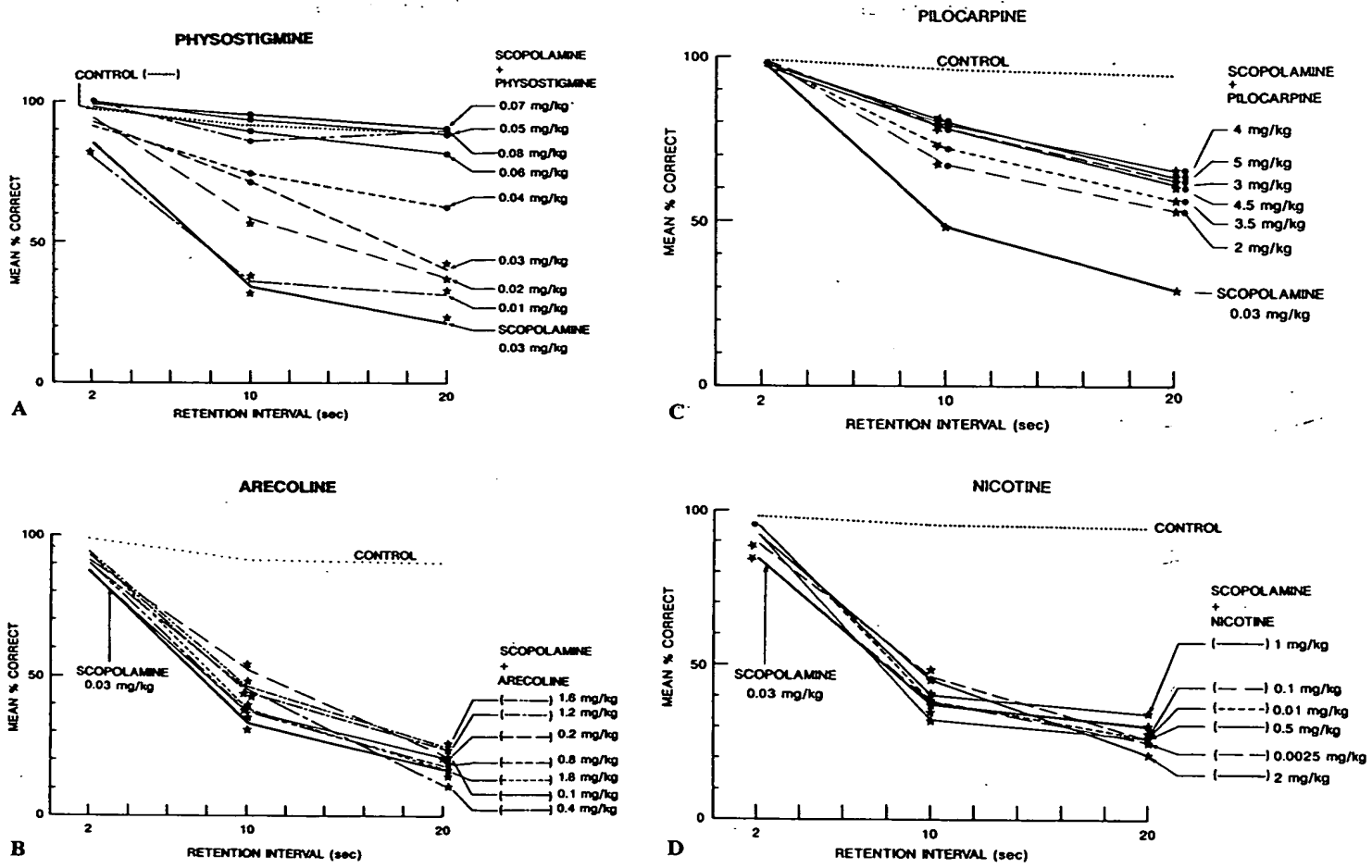

Fig. 1A–D. Effect of cholinomimetic agents on scopolamine-induced spatial memory impairment in rhesus monkeys. Test drugs were coadministered with scopolamine (0.03 mg/kg IM) 30 min prior to behavioural testing ($n=4$–6 at each dose). Data were subjected to one-way analysis of variance at each retention interval followed by one-tailed Dunnett's tests. * $P \leq 0.05$ compared to undrugged control. • $P \leq 0.05$ compared to scopolamine treatment alone. A Physostigmine 0.01–0.08 mg/kg; B arecoline 0.1–1.8 mg/kg; C pilocarpine 2.0–5.0 mg/kg; D nicotine (0.001–2.0 mg/kg)

($F=9.18$, $P<0.001$). Performance appeared to deteriorate with higher doses (0.5 and 0.75 mg/kg) which also induced marked salivation, pallor and emesis.

Nicotine. The effect of nicotine hydrogen tartrate (0.001–2.0 mg/kg IM) on recognition memory failed to reach statistical significance by repeated measures analysis of variance ($F=2.09$, $P=0.067$, Fig. 2*D*). Post-hoc one-tailed Dunnett's tests revealed that both the lowest (0.001) and the highest (2.0 mg/kg) doses tended to increase accuracy by about 10% as compared with untreated baseline values. Nicotine was well tolerated by all subjects; no adverse signs were observed at any dose examined.

Discussion

The present study has examined whether two cognitive tasks which differ in several important respects (trial-dependent versus trial-unique, spatial versus nonspatial and use of scopolamine or lists of objects to disrupt performance) are equally sensitive to the effects of cholinergic drugs in primates. We have compared an indirect agonist with directly acting agonists of differing efficacy (full or partial agonist) and selectivity (muscarinic or nicotinic).

In agreement with previous studies (Bartus and Johnson 1976) we found the disruption of spatial delayed response performance by scopolamine was greatest after long, rather than short, retention intervals. This profile has been argued to reflect a selective effect of scopolamine on mnemonic, rather than perceptual, attentional or motivational processes (Bartus and Johnson 1976). However, this interpretation has been challenged since the data may be confounded by an increase in task difficulty with longer retention intervals, and a ceiling effect is evident (Heise and Milar 1984). Therefore, the possible contribution of perceptual and attentional effects of scopolamine to this impairment cannot be ruled out. Indeed, the disruptive effects of scopolamine on visual discrimination (Evans 1975; Ksir 1975) and attention (Wesnes and Warburton 1984) using other paradigms are well established. Moreover, in marmosets, visual discrimination learning, but not retention, was impaired by scopolamine (Ridley et al. 1984).

Like Bartus (1978), we observed an impressive reversal of the disruptive effects of scopolamine by physostigmine which was clearly the most efficacious and the best tolerated agent examined, restoring performance to levels indistinguishable from undrugged animals. This effect of physostigmine seems unlikely to be due to improved retention. Thus,

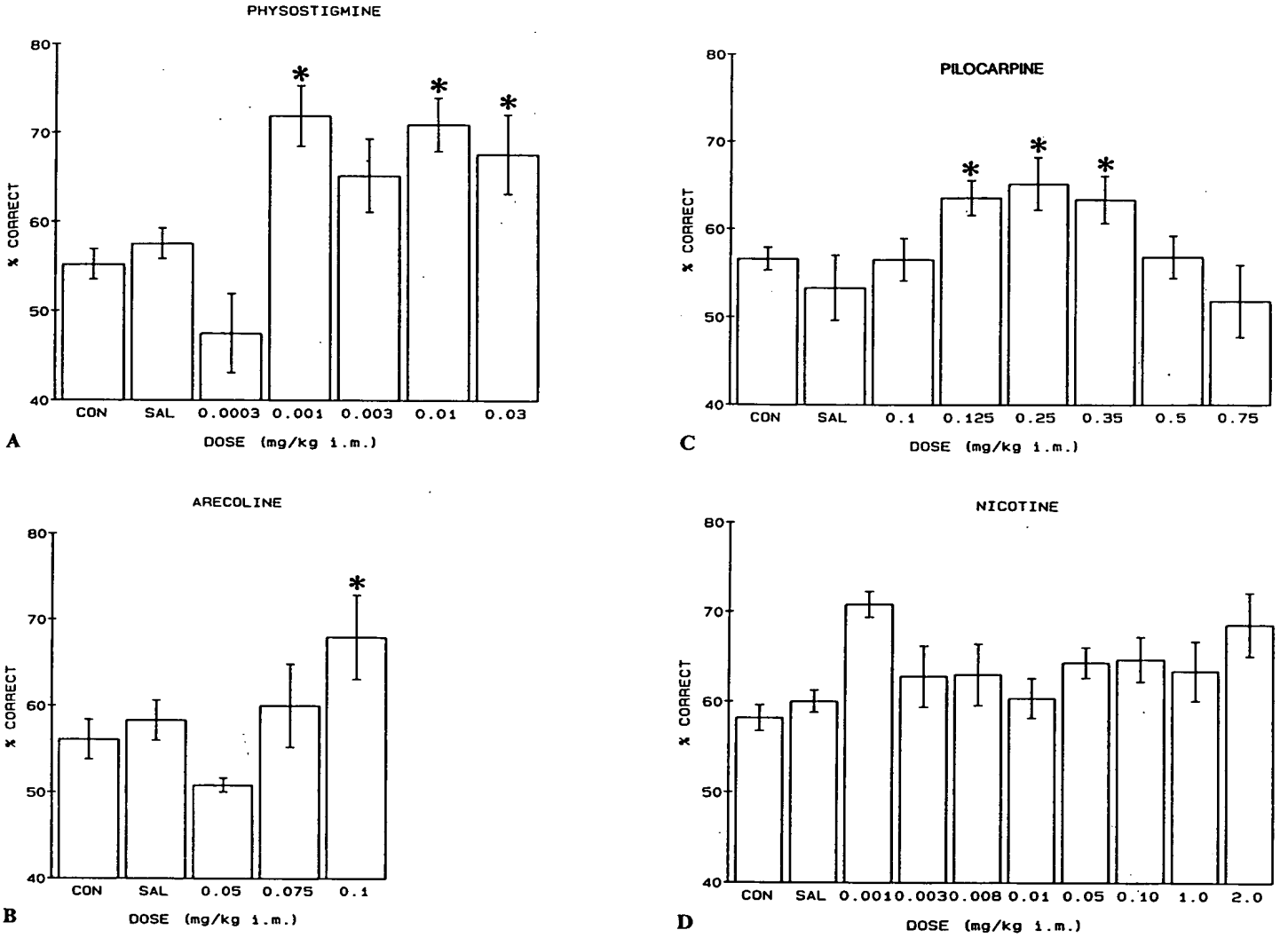

Fig. 2A–D. Effect of cholinomimetic drugs on visual recognition memory in rhesus monkeys. Test compounds were administered 20 min before testing except for nicotine, which was given 10 min previously. Each bar represents the mean ± 1 SEM for 3–4 animals. Data were subjected to one-way analysis of variance and one-tailed Dunnett's tests. * $P \leq 0.05$ compared to untreated control performance. **A** Physostigmine (0.0003–0.03 mg/kg IM); **B** arecoline (0.05–0.1 mg/kg IM); **C** pilocarpine (0.1–0.75 mg/kg IM); **D** nicotine (0.001–2.0 mg/kg IM)

aged primates exhibit an apparently similar impairment in spatial delayed response to that induced by scopolamine in young animals, but the ability of physostigmine to improve performance in old monkeys was considerably less impressive and more variable than its effects in the scopolamine model (Bartus 1979). Moreover, if in young animals the duration of the retention interval was increased until response accuracy was reduced to the levels seen in aged monkeys, physostigmine was unable to improve performance in four out of six subjects (Bartus 1979). These observations are consistent with the proposal that cholinergic drugs do not affect retention, but rather modulate discriminability at the level of perception, attention, consolidation or retrieval (Heise and Milar 1984; Sahakian 1988). In contrast, deterioration of memory clearly does occur in Alzheimer's disease (Corkin 1982; Morris and Kopelman 1986; Sahakian et al. 1988). It is not surprising, therefore, that despite the dramatic effects of physostigmine in scopolamine reversal models, improvements in cognition in Alzheimer's patients using this agent have been rather modest (Christie et al. 1981; Davis et al. 1981). A serious criticism of therapeutic screens which rely on the ability of a drug candidate to reverse the effects of scopolamine would therefore be that agents which enhance retention might not be detected. In addition, because scopolamine may affect behaviour in many ways, these screens could give rise to "active" agents which have no *direct* effect on cognition, altering some other aspects of performance instead (see Smith 1988).

For these reasons it would be advantageous to evaluate the effects of putative cognitive enhancers in tasks which are sufficiently taxing to eliminate the need to use pharmacological agents like scopolamine. The visual recognition memory test in primates which requires memory for lists of objects appears to be such a task. The ability to identify the novel objects correctly using this type of task was greatly reduced in patients suffering from Alzheimer's disease (Moss et al. 1986; Irle et al. 1988). In rhesus monkeys, the ability to recognise and avoid members of the original list was enhanced by physostigmine in a dose-dependent

manner (Aigner and Mishkin 1986; present study). Unlike the spatial delayed response task, the use of an anticholinergic agent was not required in order to reveal the effects of physostigmine using this paradigm. The reason for the improvement in performance is not known, but may be attributable to an improvement in discrimination between the stimuli at various levels of cognitive processing. It is of interest to note that although performance was improved by physostigmine, choice accuracy was not increased beyond 70% correct in the present study. This suggests that other cognitive processes (such as retention) not sensitive to cholinergic drugs were also taxed in this task, but not in the spatial task employing scopolamine. In normal human volunteers slow intravenous infusion of physostigmine was able to increase the number of words retrieved in a verbal learning task (Davis et al. 1978). In Alzheimer's patients, picture recognition (Christie et al. 1981) was also improved during intravenous or oral administration of physostigmine.

There is clearly a risk that therapeutic screens which employ scopolamine might lack sensitivity to agents which may be of use as cognitive enhancers in dementia, but which cannot, for example, displace scopolamine from its cerebral binding sites in vivo. In the present study it appeared that even for a highly efficacious muscarinic agonist like arecoline, the presence of pharmacological blockade of these sites necessitated the use of high doses of agonist which reached the toxic range before any effects on cognition could be detected. In this respect the model clearly differs markedly from the known neuropathology of Alzheimer's disease, where postsynaptic muscarinic receptors remain accessible and appear normal (Davies and Verth 1978). The inability of arecoline to reverse the effects of scopolamine following coadministration in primates in our experiments is likely to be due to the extremely short half-life and poor bioavailability of this compound in vivo. In rodents, percentage brain penetration in vivo, as assessed by inhibition of ^{3}H-oxotremorine-M ex vivo binding, is approximately 36 times lower for arecoline than for pilocarpine (Freedman et al. 1989). This problem was apparently exacerbated by the need to overcome pharmacological blockade by scopolamine in our experiments, since arecoline was able to improve spatial delayed response performance in aged primates (Bartus et al. 1980), visual discrimination learning in marmosets following lesion of the nucleus basalis (Ridley et al. 1986), position discrimination learning in marmosets treated with hemicholinium (Ridley et al. 1987), and visual recognition memory in normal young rhesus monkeys (present study). In the presence of scopolamine we were unable to increase the dose of arecoline to the level where cognitive improvement might have been detected owing to marked tremor and ataxia shortly after treatment. This failure cannot be entirely attributed to differences in pretreatment times in different studies. Thus, a period of approximately 60 min elapsed between the time of dosing and completion of behavioural testing in both the visual recognition and scopolamine reversal paradigms described here. In normal human volunteers, arecoline has been reported to improve serial learning (Sitaram et al. 1978) and word recall (Weingartner et al. 1979). Picture recognition was also improved in Alzheimer patients (Christie et al. 1981).

Unlike arecoline, pilocarpine was capable of partially reversing the effects of scopolamine, although again its effectiveness was limited by the induction of side-effects (salivation and emesis) at high doses. Similarly, pilocarpine also improved discrimination learning in marmosets treated with hemicholinium (Ridley et al. 1987), visuospatial learning in marmosets following lesions of the cholinergic projection to the hippocampus (Ridley et al. 1988) and improved choice accuracy in a visual recognition memory task in normal rhesus monkeys (present study). Pilocarpine appeared to be tolerated better than arecoline, possibly because of its relatively low efficacy as a muscarinic receptor agonist (Freedman et al. 1988). In view of these findings, further clinical evaluation of pilocarpine in senile dementia might be warranted. Although in the single clinical trial so far carried out with pilocarpine no improvement in cognition was observed, only two patients with suspected Alzheimer's disease were studied, and only one dose of pilocarpine (Caine 1980).

Compared with the effects of muscarinic agonists, the ability of nicotine to improve performance in the present studies was not impressive. Nicotine appeared to attenuate the disruption of performance induced by scopolamine which occasionally occurs even after short retention intervals. However, there was no reversal of the marked effects of scopolamine after longer delays. Nor was there evidence for a robust effect of nicotine on visual recognition memory. Like Elrod et al. (1988), who used a delayed colour matching task in monkeys, we observed considerable variability in the response of different individuals to the effects of nicotine, and doses which appeared to improve performance in some animals failed to induce a significant effect across the group. Human volunteer studies have indicated improvements in rote learning (Anderson and Post 1974) and rapid information processing (Wesnes and Warburton 1984) following cigarette inhalation. These effects may be due to reversal of the effects of fatigue on performance. Alzheimer patients appear more sensitive to the effects of nicotine than normal volunteers, and showed cognitive improvement in a very narrow dose window (0.25 μg/kg/min IV; Newhouse et al. 1988).

In conclusion, we have demonstrated beneficial effects of full and partial muscarinic agonists in two primate cognitive tasks. The visual recognition task offers several advantages over a scopolamine-reversal model for the detection of novel therapeutic agents for Alzheimer's disease.

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Original Investigations

Studies on Human Memory: The Interactions of Diazepam, Scopolamine, and Physostigmine

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Abstract. Seventy volunteers were injected with diazepam (0.3 mg/kg), scopolamine (8 µg/kg), or placebo, followed 70 min later by another injection of physostigmine, physostigmine and methscopolamine (in case of diazepam treatment), or placebo. Physostigmine was given in two doses, 16 and 32 µg/kg; methscopolamine, 8 and 16 µg/kg. Subjects (*Ss*) were tested in groups of 5 in a double blind procedure with treatments distributed according to a Latin square design. Prior to treatment, *Ss* heard a series of lists of words, followed by an immediate recall test. Following the first injection, delayed free recall and recognition tests were given. The second drug was then injected, followed by a presentation of another two sets of lists which were tested similarly. Subjective feelings were also evaluated with a rating questionnaire.

Diazepam and scopolamine did not affect recall of information which had been learned prior to drug injection. However, both drugs impaired the learning or acquisition of new information. Physostigmine, especially in its high dose, antagonized most of the memory deficits produced by scopolamine while those of diazepam remained. This is a strong indication that scopolamine acts centrally through an anticholinergic mechanism while diazepam may act through a different system.

Key words: Diazepam – Scopolamine – Physostigmine – Memory – Drug interactions.

Numerous animal experiments have been interpreted as indicating that memory is mediated by changes at cholinergic synapses. Kerkut et al. (1970) observed in cockroaches a rapid fall in acetylcholinesterase activity of the metathoracic ganglia during learning. Anticholinesterases injected before training enhanced learning with a dose-response effect. Deutsch (1971) concluded that the cholinergic system is implicated in information storage mechanisms. Mandel and Ebel (1974) found a highly significant cholinergic enzyme activity in the temporal lobes of a strain of mice characterized by high levels of avoidance and maze learning as compared to a strain with poor avoidance and maze levels.

In a previous experiment (Ghoneim and Mewaldt, 1975), we demonstrated that both scopolamine and diazepam (Valium) impair human memory in a specific and similar manner. Scopolamine is a well-known competitive antagonist of acetylcholine at the receptor sites on the postsynaptic membrane. Contrary to its peripheral effects, the mechanism of its central nervous system effects are not well understood and may reflect pharmacologic actions other than cholinergic blockade (Greenblatt and Shader, 1973). Certain findings in animals suggest that diazepam produces relatively selective depression of limbic system activity (Schallek et al., 1964), possibly by an anticholinergic action (Consolo et al., 1975). The increase in brain acetylcholine accompanied by a lack of effect on choline levels, choline acetyltransferase, and cholinesterase activities led Consolo et al. (1974) to postulate that the drug may act by blocking the release of acetylcholine from preganglionic nerve terminals.

In this study we hoped to determine whether both scopolamine and diazepam affect human memory through an anticholinergic action by attempting to reverse their effects through the use of physostigmine (Antilirium), an inhibitor of acetylcholinesterase. The effects of anticholinergic drugs should be opposite to those of cholinesterase inhibitors, provided that the actions concerned are mediated through the cholinergic system.

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METHOD

Subjects. Thirty-five male and 35 female university students served as paid volunteers. Their ages varied from 19 to 32 with a mean age of 23 years. An informed consent for participation in the study was obtained from each *S*, although to avoid bias the names of the drugs were not revealed.

Drugs. The following drugs and doses were administered as listed: diazepam (0.3 mg/kg) intravenously, scopolamine (8 μg/kg) intramuscularly, physostigmine (16 and 32 μg/kg) intramuscularly, methscopolamine (Pamine) (8 and 16 μg/kg) intramuscularly, and saline (placebo) intramuscularly.

Design and Tests. Subjects were tested in groups of five and were assigned to conditions in an unbiased fashion. All *Ss* in each experimental session had the same treatment. There were seven treatments (Table 1), for each of which ten subjects were allocated, with both sexes equally represented. Treatments were distributed according to a Latin square design.

A training session was carried out before starting the actual experiment to familiarize the *Ss* with the experimental tasks. Each of the following tests were administered in the order shown in Table 2. The tests were administered by a person unfamiliar with the identity of drugs given.

Subjective Rating Questionnaire. Subjects rated their feelings on 16 scales by drawing a perpendicular line across a horizontal, unmarked 100 mm line connecting two adjectives representing the extremes of the condition to be rated. The position of the vertical line was measured in millimeters and used as the score. The 16 adjective pairs fell into each of four categories of feelings: mental sedation (e.g., alert-drowsy, fuzzy-clear headed), physical sedation (e.g., strong-weak, well coordinated-clumsy), transquilization (e.g., calm-excited, troubled-peaceful), and attitudes or other feelings (e.g., happy-sad, interested-bored). The scales were derived from Norris (1971).

Immediate Recall Tests of Lists in Set I. Subjects listened to eight lists of 16 words each. The lists were presented by a tape recorder at a rate of one word every 2 s. Immediately after presentation of each list, *Ss* were to recall in any order as many words as they could remember from the list they had just heard. A period of 1.5 min was allowed for the written recall of each list. Three lists of names were used for the practice session, while the rest of the lists consisted of nouns selected to have a frequency of 10–40 per million according to Thorndike and Lorge (1944).

Delayed Free Recall Test of Lists in Set I. Subjects were asked to write as many words as they could remember from the set of eight lists they had learned prior to injection. Twenty minutes were allowed for the recall session.

Delayed Recognition Tests of Lists in Set I. A booklet containing 128 pairs of words was given to each *S*. One word of each pair was "old," i.e., it occurred previously in one of the 8 lists, while the other word was "new," i.e. it was not previously presented. Subjects were asked to check the word in each pair which they had heard before. Ten minutes were allowed for this test. (Note: Subjects had not previously been informed that original learning would be tested with either delayed recall or recognition and neither test was included in the practice session.)

Immediate, Delayed and Recognition Tests of Lists in Set II. A second set of 8 lists identical in construction to the first were learned and tested with the same procedures described above. Words on the new lists and foils on the recognition tests had not been employed on the earlier lists. In delayed recall, *Ss* were asked to recall only words from the most recent set of lists.

Categorized vs. Noncategorized Immediate, Delayed and Recognition Tests. A third set of lists were learned and tested as described above; however, in this set only four 16-item lists were employed. Lists 1 and 4 were identical in construction to the previous lists while lists 2 and 3 were constructed so as to contain four high-frequency instances of four different semantic categories each as taken from the Battig and Montague norms (1969). For example, one category was fruit from which peach, apple, banana, and orange were chosen items. Items within a category were randomly placed throughout the list and *Ss* were not informed about the categorical composition of these lists.

Table 1. Treatment groups

First drug	Second drug
1. Diazepam	placebo
2. Diazepam	physostigmine and methscopolamine[a] (low doses)
3. Diazepam	physostigmine and methscopolamine[a] (high doses)
4. Placebo	placebo
5. Scopolamine	placebo
6. Scopolamine	physostigmine (low dose)
7. Scopolamine	physostigmine (high dose)

[a] The two drugs were injected separately

Table 2. Scheme of the experimental procedures

Elapsed time since first drug administration[a]	Experimental procedure
	Subjective questionnaire test
	Practice session
	Presentation and immediate recall of first set of lists
	First drug or placebo administration
30 min	Subjective questionnaire test
35 min	Delayed recall test of first set of lists
55 min	Delayed recognition of first set of lists
70 min	Second drug or placebo administration
90 min	Presentation and immediate recall of second set of lists
110 min	Subjective questionnaire test
115 min	Delayed recall of second set of lists
135 min	Delayed recognition of second set of lists
145 min	Presentation and immediate recall of third set of lists
155 min	Rest period
160 min	Delayed recall of third set of lists
170 min	Delayed recognition of third set of lists

[a] Minutes from first drug administration to the beginning of each test

Table 3. Mean number of words recalled and their corresponding standard errors for immediate and delayed recall and recognition tests for lists in Set I and Set II

		Set I			Set II		
		Immed. recall	Delayed recall	Recognition	Immed. recall	Delayed recall	Recognition
Diazepam – placebo	Mean	65.90	37.80	111.50	54.00	14.80	99.00
	SE	6.98	7.91	5.01	5.73	6.85	5.52
Diazepam – physostigmine[1]	Mean	60.70	30.20	106.60	47.10	10.90	98.30
	SE	4.09	4.45	3.37	3.49	2.39	3.06
Diazepam – physostigmine[2]	Mean	72.60	41.00	116.00	53.70	15.20	103.50
	SE	5.09	5.33	2.21	4.79	3.96	2.30
Placebo – placebo	Mean	70.40	34.60	110.20	70.60	32.70	112.10
	SE	3.36	4.43	2.99	3.35	3.61	2.12
Scopolamine – placebo	Mean	72.80	40.60	112.30	39.10	12.30	94.70
	SE	7.04	7.55	2.90	6.21	5.32	4.44
Scopolamine – physostigmine[1]	Mean	68.10	33.70	114.10	50.40	17.50	107.00
	SE	4.69	3.49	2.46	3.64	2.80	3.60
Scopolamine – physostigmine[2]	Mean	74.50	39.60	109.70	55.80	20.10	106.90
	SE	3.40	3.17	2.42	2.82	3.19	2.75

Physostigmine[1] = 16 μg/kg
Physostigmine[2] = 32 μg/kg

RESULTS

The results of the first two sets of memory tests were analyzed by means of a 2×7 (sex × drug) analysis of variance. The means and standard errors for immediate and delayed recall, and for the recognition tests on these two sets of tests are presented in Table 3.

Immediate Recall of Lists in Set I. To assure that the groups did not differ in learning ability before injection, the total number of correct responses summed across lists was analyzed. As anticipated, no significant group differences were found ($F < 1$). However, as in a previous study (Ghoneim et al., 1975), there was a large sex effect, $F(1,56) = 8.85$, $P < 0.01$. Mean recall for female *Ss* was 74.8 items correct, compared to 63.8 correct for the males. This sex effect was also observed on the subsequent memory tests; however, because sex never interacted with any drug treatment, it will not be reported further.

Delayed Recall and Recognition of Lists in Set I. Following injection, retention of the first set of words was again tested. As with the immediate recall tests, the analysis for both the delayed recall and recognition tests indicated no significant drug effects nor interactions ($F < 1$ in all cases).

Immediate Recall of Lists in Set II. Analysis of immediate recall of lists learned after drug administration indicated a large drug effect, $F(6,56) = 4.32$, $P < 0.001$. Follow-up analyses indicated that all drugged groups recalled significantly fewer words than the placebo group ($P < 0.05$ in each case). In addition, the group given scopolamine-placebo performed significantly worse than each of the diazepam groups, ($P < 0.05$). More interestingly, however, the group given scopolamine followed by the large dose of physostigmine recalled significantly more words than the scopolamine-placebo group ($P < 0.05$), and in the group given scopolamine followed by the low dose of physostigmine, the recall differences from the scopolamine group approached significance ($0.05 < P < 0.1$).

Delayed Recall and Recognition of Lists in Set II. A pattern of results similar to that on the immediate recall test was observed in delayed recall. Again, there was a strong drug effect, $F(6,56) = 3.03$, $P < 0.05$. This time, however, while performance in each of the drug groups was significantly poorer than in the placebo groups, $P < 0.05$ at least in each case, the scopolamine-placebo groups versus scopolamine followed by the large dose of physostigmine group comparison only approached significance ($0.05 < P < 0.1$).

The recognition test also indicated that the drugs were affecting performance, $F(6,56) = 3.41$, $P < 0.1$. In this test, however, while the placebo group performed significantly better than each of the diazepam groups and the scopolamine-placebo group, $P < 0.05$, it did not differ significantly from either of the scopolamine-physostigmine groups. In addition, both of the

Table 4. Mean number of words recalled and their corresponding standard errors for immediate and delayed recall and recognition tests for lists in Set III

Treatment with			diazepam-placebo	diazepam-physostigmine[1]	diazepam-physostigmine[2]	placebo-placebo	scopolamine-placebo	scopolamine-physostigmine[1]	scopolamine-physostigmine[2]
Immediate recall	categorized lists	Mean	22.30	22.00	21.60	26.00	15.80	19.30	22.30
		SE	1.38	0.91	1.65	1.06	2.08	1.52	1.41
	non-categorized lists	Mean	13.80	12.80	13.10	17.20	9.60	9.80	12.20
		SE	1.75	0.81	1.43	0.74	1.62	1.04	1.18
Delayed recall	categorized lists	Mean	14.10	14.60	13.00	23.00	9.20	12.30	15.70
		SE	2.76	1.91	2.40	1.70	2.22	1.85	1.91
	non-categorized lists	Mean	5.40	2.60	4.40	5.50	2.60	2.10	3.70
		SE	2.21	0.66	1.76	0.84	1.15	0.55	0.55
Recognition	categorized lists	Mean	26.50	26.60	26.40	30.10	24.20	26.30	28.70
		SE	1.22	1.32	1.02	0.30	1.48	1.55	0.60
	non-categorized lists	Mean	27.30	26.00	25.20	28.70	26.30	27.30	26.40
			1.11	0.71	0.58	0.55	1.08	0.26	0.95

Physostigmine[1] = 16 µg/kg
Physostigmine[2] = 32 µg/kg

scopolamine groups which received physostigmine performed significantly better than the scopolamine placebo group, $P < 0.05$.

Performance on Lists in Set III. The results of each test in Set III were analyzed in separate $2 \times 2 \times 3$ analyses of variance with list type (categorized or noncategorized) as a within-*S* factor, and sex and drug as between-*S* factors. The data is summarized in Table 4. The results of the analysis of immediate recall indicated strong main effects for both the list type and drug variables, $F(1,56) = 348.74$, $P < 0.001$ and $F(6,56) = 6.03$, $P < 0.001$ respectively. Mean recall for categorized lists was 21.3 items correct versus 12.6 items correct for the noncategorized lists. Follow-up analyses of the significant drug effect indicated that both the scopolamine-placebo and the scopolamine followed by the low dose of physostigmine groups still showed significant impairment from the placebo group and other drug groups, but all other drug treatments did not differ significantly from the placebo group or from each other. However, the comparison of each of the drug treatments to the placebo approached significance, $P < 0.1$.

In delayed recall, again there was a powerful list type and drug effect, $F(1,56) = 265.03$, $P < 0.001$ and $F(6,56) = 3.96$, $P < 0.01$. In addition, there was a significant Drug × List Type interaction, $F(6,56) = 4.06$, $P < 0.01$. Schéffe contrasts indicated that recall of words from non-categorized lists did not differ between groups, $P > 0.1$. However, for the categorized lists, the placebo group recalled significantly more words than any of the drug groups, $P < 0.05$. In addition, the scopolamine-placebo group recalled significantly fewer categorized words than the scopolamine group receiving the high dose of physostigmine, $P < 0.05$. On the recognition test, as expected, categorization produced no significant effects, $F < 1$. However, drug-produced differences were still evident, $F(6,56) = 3.56$, $P < 0.01$. The placebo group exceeded the performance levels of the two diazepam groups receiving physostigmine and of the scopolamine-placebo group, $P < 0.05$, but no other differences were significant.

Subjective Questionnaire. Because of the large amount of variability in the subjective ratings, the data were analyzed by means of an analysis of covariance. In the first set of analyses, scores following administration of the first drug were adjusted by the scores on the pre-drug questionnaire. For the second set of analyses, scores following second drug administration were

adjusted by the first drug scores. Results for individual adjective pairs were combined in order to analyze the effects of each of the four categories of feelings described earlier.

The first analyses indicated that both diazepam and scopolamine produced marked mental and physical sedation compared to placebo, $P < 0.001$. However, the effect of the two drugs did not differ significantly. In addition, contrary to scopolamine, diazepam produced greater feelings of tranquilization than placebo, $P < 0.01$. For the second set of analyses, the large dose of physostigmine increased the feelings of mental and physical sedation produced by diazepam and increased the ratings of being troubled and tense in the same group, $P < 0.01$. The effects of physostigmine in those subjects who received scopolamine did not reach statistical significance, although their direction was opposite to those of the diazepam group.

DISCUSSION

The doses of the drugs used were within the therapeutic range. The manufacturer of physostigmine recommends a dose twice that of the anticholinergic drug whose action is to be reversed. Our high dose of physostigmine was four times that of scopolamine. We did not want to use a higher dosage because of the danger of inducing a cholinergic crisis. The doses of scopolamine and diazepam were chosen on the basis of previous studies which demonstrated a strong effect on memory. The time of administration of physostigmine was also chosen on the basis of previous work by Crowell and Ketchum (1967). They reported that physostigmine was more effective in treating scopolamine-induced delirium the longer the time span after scopolamine administration.

Since diazepam does not inhibit the peripheral effects of physostigmine, we administered methscopolamine bromide simultaneously with physostigmine when diazepam was the first drug administered. Methscopolamine, a quaternary ammonium compound, induces the same peripheral actions as scopolamine, but does not exert central nervous system effects because of limited ability to cross the blood-brain barrier. We first tried a ratio of physostigmine: methscopolamine of 4:1. The first 5 subjects to whom we administered this combination suffered from frequent nausea and vomiting which interrupted the experiment, and these results were excluded from the study. We thereafter used a ratio of 2:1 for the two drugs. Drachman and Leavitt (1974) demonstrated that methscopolamine produces no significant effects on memory nor cognitive functioning and therefore should not have interfered with any physostigmine-reversal of the memory effects of diazepam.

The results for the first set of lists indicate that scopolamine and diazepam did not affect recall of information which had been learned prior to drug injection. The absence of differences between the drug and placebo groups on the delayed recall and recognition tests suggests that scopolamine and diazepam do not significantly influence the retrieval of information from memory if that information is adequately stored. The results for the second set of lists when placebo was the second injection clearly demonstrate that performance on material learned after the administration of diazepam or scopolamine was markedly impaired. Since retrieval was not impaired and learning deficits were evident in the immediate recall tests following injection, it would appear that diazepam and scopolamine primarily interfere with the storage process, i.e., they impede the transfer of information from the short-term to the long-term memory store (Atkinson and Shiffrin, 1968 and 1971). This is all in agreement with the results from a previous study (Ghoneim and Mewaldt, 1975). It is interesting that the learning deficit produced by the 2 drugs is similar in form to, though of lesser magnitude than, that seen in patients with Korsakow's psychosis (Baddeley and Warrington, 1970), patients who suffer bilateral lesions of the hippocampus (Penfield and Mathieson, 1974), and to the memory impairment in old age (Drachman and Leavitt, 1974).

Physostigmine, especially in its high dose, antagonized most of the memory impairment produced by scopolamine. The improvement produced by physostigmine was particularly apparent in the recognition test of the second set of lists where the cues provided helped what appeared to be submaximal recovery from the effects of scopolamine. On the third set of lists, the antagonistic effect of physostigmine was still apparent. In contrast, physostigmine failed to reverse the memory deficits produced by diazepam. It also increased the physical and mental sedation produced by diazepam. It is interesting that in a recent study, physostigmine failed to counteract the peak behavioral effects of tetrahydrocannabinol and at the same time amplified lethargy and somnolence (Freemon et al., 1975). It is unlikely therefore that this is a chance observation, though its nature and mechanisms are not clear since physostigmine was not evaluated on its own. Although physostigmine is recommended as an antidote in cases of anticholinergic intoxication, for both its central and peripheral effects, the volunteers did not experience a significant amelioration of the physical and mental sedation produced by scopolamine. The improvement in memory functions produced by physostigmine in subjects who received

scopolamine seems therefore to be specific and unrelated to behavioral changes or general arousal effect.

The antagonistic effect of physostigmine on the memory deficit produced by scopolamine is a strong indication that the latter drug acts centrally through an anticholinergic mechanism. By contrast, the total lack of physostigmine antagonism of diazepam should be taken as evidence against such hypothesis for this drug. Several mechanisms for the central action of diazepam have been postulated, including the cholinergic system. New evidence, however, strongly suggests that its action is mediated through a facilitation of GABA-ergic transmission (Costa et al., 1975). There is also evidence that GABA-ergic system may play an essential role in learning (Ishikawa and Saito, 1975).

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The effects of scopolamine on working memory in healthy young volunteers

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Abstract. Twenty healthy young adults completed a series of nonverbal and problem solving tasks in a repeated measures design involving placebo and 0.6 mg scopolamine, administered by subcutaneous injection. Subjects completed the test battery under standard presentation conditions and with concurrent articulation, which precludes verbal recoding of test material. Under standard presentation conditions, scopolamine significantly impaired performance on the problem solving task and on tasks of visuo-spatial and spatial memory; memory for abstract shapes was not impaired. Concurrent articulation impaired performance on the shape recognition and interacted with drug treatment on the problem solving task. The results suggest that scopolamine impairs working memory, and that the decrement is at the level of the central executive mechanism rather than the subsystems which it controls.

Key words: Scopolamine – Cholinergic system – Cognition – Working memory – Man

The effects of scopolamine on human memory and information processing have been studied extensively over the last 15 years and recent interest in the possible link between the cholinergic system and Alzheimer's disease has provided a new impetus for more detailed investigations (Kopelman 1987; Sahakian 1987). In general, the drug administration studies suggest no effect of scopolamine on either the digit span or the short term memory component of recall, with long term recall (Ghonheim and Mewaldt 1975, 1977; Frith et al. 1984) and acquisition of information (Peterson 1977) being the primary locus of drug-induced impairments. Effects at retrieval have also been indicated in studies involving both recognition paradigms and cued recall (Ghonheim and Mewaldt 1975; Caine et al. 1981; Dunne and Hartley 1985). Kopelman (1986) concluded that scopolamine does not affect either short term memory or working memory, although these two concepts are represented by quite different experimental paradigms.

The concept of working memory developed by Baddeley and Hitch (1974) and Baddeley (1986) centres on a "central executive mechanism" which is responsible for processing and for allocation of processing resources to the task in hand. The central executive mechanism is aided by two systems whose function is essentially maintenance of information: the articulatory loop and the visuo-spatial system. As the names suggest, these handle specific types of information, with the former exclusively used for verbal material and the latter for nonverbal information. The work of Baddeley and Hitch (1974) specifically dissociated the short term component of free recall and the digit span from the central functioning of the working memory system which is implicated in the everyday tasks of holding, sorting and processing information and generally liasing between incoming information and retrieval from semantic memory. The subcomponents of the working memory system, the central executive mechanism, the articulatory loop and the visuo-spatial scratch pad, can be dissociated using different tests and by the task-specific interference which can be generated. For example, Baddeley and Hitch (1974) demonstrated that if the articulatory loop is "loaded" by requiring subjects to maintain a sequence of digits, the loop is disabled and processing efficiency is impaired for tasks which involve verbal coding and maintenance. Tasks which do not utilise the articulatory loop, for example tasks of nonverbal memory, would be unaffected by such loading.

A number of studies of patients with Alzheimer type dementia, a disease which is thought to involve a substantial loss of brain acetylcholine neurons, have used the working memory model to identify specific patterns of memory deficits associated with the disease. Morris (1984, 1986) reported that although Alzheimer patients are significantly poorer on digit span tasks, there was no evidence for impairments on tasks involving the articulatory loop, but only on short term memory tasks having a heavier processing load. Baddeley et al. (1986) found Alzheimer patients were severely impaired in a dual-task experiment requiring divided attention. The implication from these studies is of impairments in central executive function for Alzheimer patients. Recent work by Sahakian et al. (1987) reports that such patients are also poorer than controls on nonverbal tasks of pattern and shape recognition and visuo-spatial learning, tasks which presumably employ the visuo-spatial system. The traditional tests of short term memory which have been used in drug studies to date are primarily associated with the articulatory loop (e.g., digit span: Drachman and Leavitt 1974; Mohs and Davis 1985; recency effects in list recall: Crow et al. 1975; Mewaldt and Ghonheim 1979). In this study, we have examined the effects of scopolamine in healthy young adults on a series of tasks which have been used with Alzheimer patients and which are associated with the different components of working

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memory. The aim was to examine the comparability of the clinical and drug-induced deficits in the light of a conceptually more specific model of memory.

The tests assessed problem solving, pattern recognition, memory for spatial location and visuo-spatial learning. In order to isolate the contribution of the articulatory loop in these tests, and to specify the effects of cholinergic blockade more clearly, the tests were completed under standard conditions and with concurrent articulatory activity, where subjects completed the tasks while repeating aloud a 4-digit number at a regular and continuous pace[1]. This verbal activity precludes verbal recoding of information from the visually presented test sets for maintenance in the articulatory loop during task completion (Salame and Baddeley 1982).

If subjects commonly make use of the articulatory loop to facilitate processing, disabling that loop will increase the processing load of the central executive mechanism. If the effects of scopolamine are specific to the central executive mechanism, the decrements in performance following scopolamine should increase under conditions which increase the processing load of that mechanism.

Method

Subjects. Twenty healthy male undergraduate volunteers were paid for their participation in the study. They ranged in age from 18 to 29 years, and in weight from 60 to 80 kg. All volunteers underwent a full medical prior to the study; any subjects who were taking or had taken any centrally acting medications in the 2 weeks prior to the study were excluded from the study. All subjects were given three separate practice sessions prior to the onset of the study proper. The study was considered and approved by the University Ethics Committee.

Materials. BBC Master computers in individual experimental rooms presented each of the four tasks and collected the data generated by each subject.

A subset of the CANTAB computerised nonverbal test battery (Morris et al. 1987) was modified for use in this study. The tests included pattern recognition, memory for spatial location and a test of visuo-spatial memory. The tasks make use of touch sensitive screens, with subjects indicating their selection by touching the appropriate item or position on the screen.

In the shape recognition test, subjects were shown a 12-item set of shapes which appeared consecutively on the screen. At the end of the set, 12 pairs of items were presented in a two-alternative forced-choice recognition task for items presented in the previous set. The sequence was then repeated with 12 more items. For this study, random shapes were generated by a computer program, with the aim of minimising meaningful verbal coding of the shapes. Non-target items for the recognition phase were derived from the test items by systematically adding to or removing sections of the original shape.

For the spatial recognition task, the program presented a five-item sequence of boxes, each in a different location on the screen. This was followed by a five-trial recognition phase in which subjects selected the correctly located box from a choice of two locations. There was a total of four novel sequences in this task.

In the delayed response test of visuo-spatial memory, subjects are required to remember the locations of abstract visual stimuli hidden in a set of eight boxes. Each box opened in turn to reveal the shape inside. Then the shapes appeared in a different random order in the centre of the screen and subjects indicated the box in which each had previously appeared. The sequence of presentation and test trials was repeated until the subject remembers the correct location of all eight items on a single trial; items were presented in a different random order on all presentation and test sequences. The original program for this test was modified so that all test items occurred in the same colour, in order to assess memory for location of each shape in the absence of colour cues.

Problem solving skills were examined using the Baddeley Logic task (Baddeley 1968). In this task, a sentence describing the order of two letters (e.g., A follows B) was presented on the screen along with the two letters (e.g., AB). Subjects were required to evaluate the veracity of the sentence as a description of the order of the letters and to indicate their decision by pressing the appropriate response key (YES for correct order, NO for incorrect). "Yes" and "no" responses and reaction times to make those responses were recorded for each sentence. The response to the sentence initiated immediate presentation of the next problem in the series. There were 64 sentences involving four different grammatical constructions; presentation order of the sentences was random, and three different letter pairs (AB, FG and PQ) were used in the three test sessions.

Design and drug. A dose of 0.6 mg scopolamine or placebo was administered subcutaneously. Subjects received the drug and the placebo each on two separate occasions in a repeated measures design across four experimental sessions, each one a week apart. Order of treatment was counterbalanced across the sessions such that a subject received one drug treatment in each half of the study. Treatments were administered double blind.

Procedure. Each session lasted 5 h, incorporating three 45-min periods of testing. Subjects were given a light breakfast on arrival at the unit, and this was followed by a baseline test (T0) on each of the tasks included in the battery. Drug treatment was administered on completion of baseline tests. The test battery was then repeated at 1 h (T1) and 2 h 20 min (T2) after drug. In each case, parallel unrepeated forms of the tasks were used, administered in the same order on each occasion, with the Logic task preceding the nonverbal test battery.

In weeks 3 and 4, subjects completed all tests under conditions of articulatory suppression. They were required to repeat a 4-digit number at a constant rate throughout the completion of each of the tasks. The number was given to the subject before the task commenced, and he was instructed to establish a regular and comfortable pace in his

[1] An alternative procedure would have been to "load" the articulatory loop by requiring subjects to maintain a set of digits throughout completion of the task. This procedure is effective only if the digit sequence is near span, since the principle is to fill the loop to prevent test items being maintained in it. Concurrent articulation disables the loop by preventing the verbal recoding required to input the material into an articulatory system, and for visually presented test material serves essentially the same purpose as a concurrent memory load.

repetition before initiating the test; the experimenter ensured that the pace was sufficient to prevent any simple time-share strategy. The subject was informed that the number would be checked for accuracy at the end of each test. Subjects had had no practice in completing the tasks under conditions of articulatory suppression.

Results

Statistics

Results were analyzed separately for the two conditions of testing (standard versus articulatory suppression) at each time period (baseline, T1 and T2), using a two-way analysis of variance with treatment as a within-group factor and order of administration as a between-group factor (Kirk 1982). An additional analysis was completed to compare the drug treatment across the two presentation conditions, with treatment and test condition (standard versus articulatory suppression) as within-subject factors and order of treatment as a between-subject factor. As the SUPPRESSION condition was run in the final 2 weeks of the study, the order factor in the analysis is not strictly a measure of session effects, as has been assumed in the individual ANOVAs, and interpretation of order effects should be conservative. The main purpose of this combined ANOVA was to establish the effects of articulatory suppression. In this respect, the relative order in which the conditions were presented means that any practice effects still occurring across sessions will reduce the potentially disruptive effects of articulation, and will reduce the danger of incorrectly rejecting the null hypothesis.

Unless stated, there were no differences between groups on baseline measures.

Baddeley logic task

a. Standard presentation. Response times decreased across sessions for both treatment groups, producing a significant order × treatment interaction on the baseline (T0) measures [$F(1,18)=5.64$, $P<0.03$].

The effects of drug on performance were not statistically significant one hour after administration. At T2, 2 h 20 min after treatment, scopolamine significantly decreased the number of correct responses [$F(1,18)=7.09$, $P<0.02$; Fig. 1a], and slowed reaction times for correct responses [$F(1,18)=6.5$, $P<0.02$; Fig. 2a]. However, this latter effect was qualified by an interaction with order [$F(1,18)=5.07$, $P<0.04$]; subjects who received scopolamine in the first session were slower than all other groups, who did not differ across treatment conditions or sessions.

b. With concurrent articulation. Baseline measures revealed significant improvements in correct evaluations for both groups of subjects across sessions [$F(1,18)=12.99$, $P<0.002$], demonstrating a practice effect under these conditions.

At T1, there were significant effects of treatment on number correct [$F(1,18)=8.78$, $P<0.008$], with scopolamine impairing performance. Reaction time to correct responses decreased across sessions independent of treatment [$F(1,18)=8.53$, $P<0.01$].

At T2, the same pattern was observed. Scopolamine significantly lowered performance in terms of number correct [$F(1,18)=9.49$, $P<0.006$], and response times decreased across sessions independent of treatment [$F(1,18)=4.90$, $P<0.04$]. These effects are shown in Figs. 1b and 2b.

In order to examine the effect of concurrent articulation, a three-way ANOVA was completed, with condition as a repeated measure. Baseline reaction times to make correct

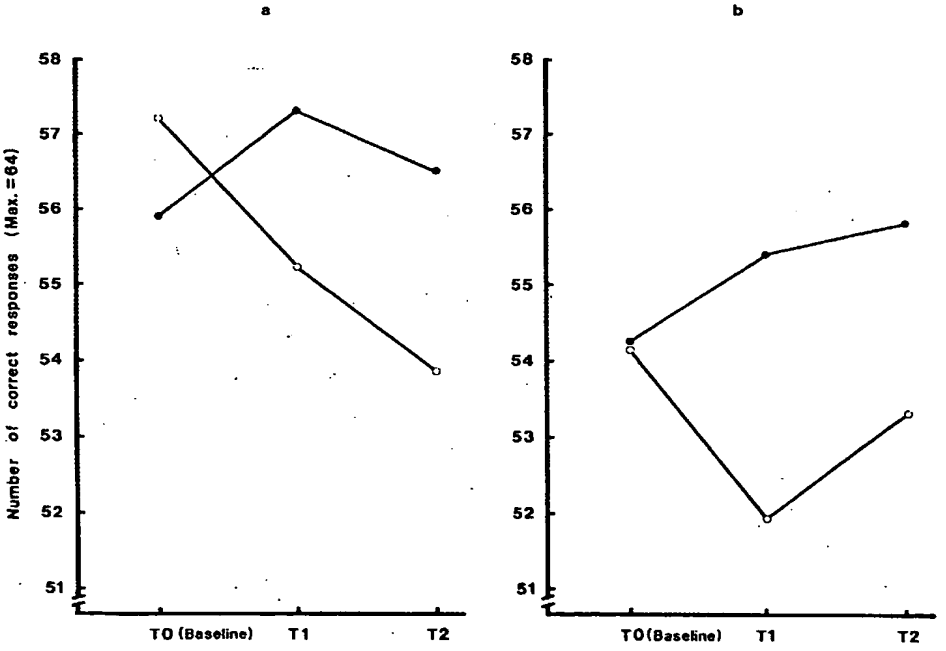

Fig. 1a, b. Number of correctly evaluated sentences in the Baddeley Logic task with a standard presentation and b concurrent articulation of a 4-digit number. ●—● Placebo; ○—○ scopolamine (0.6 mg)

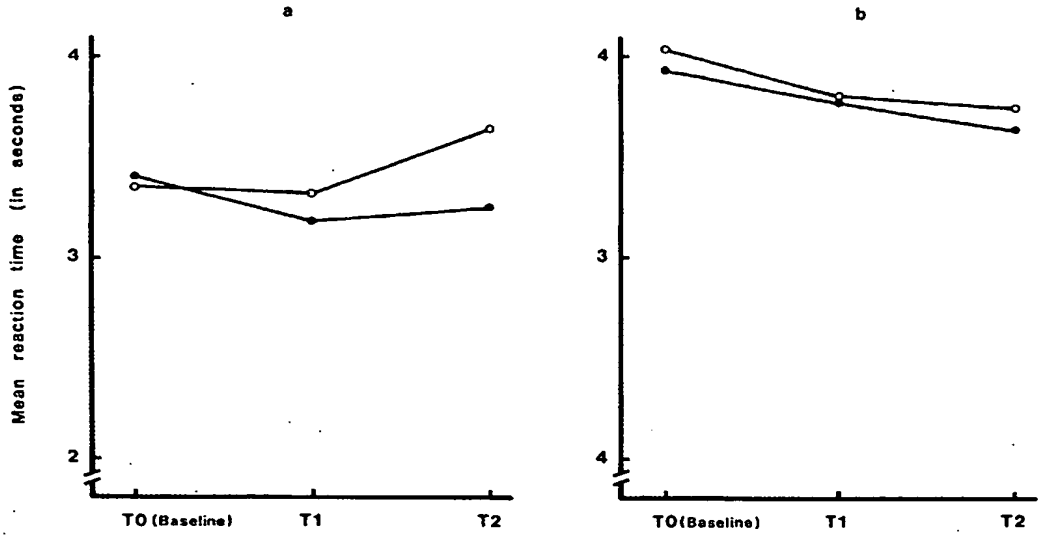

Fig. 2a, b. Mean response times for correctly evaluated sentences in the Baddeley Logic task with a standard presentation and b concurrent articulation of a 4-digit number. ●—● Placebo; o—o scopolamine (0.6 mg)

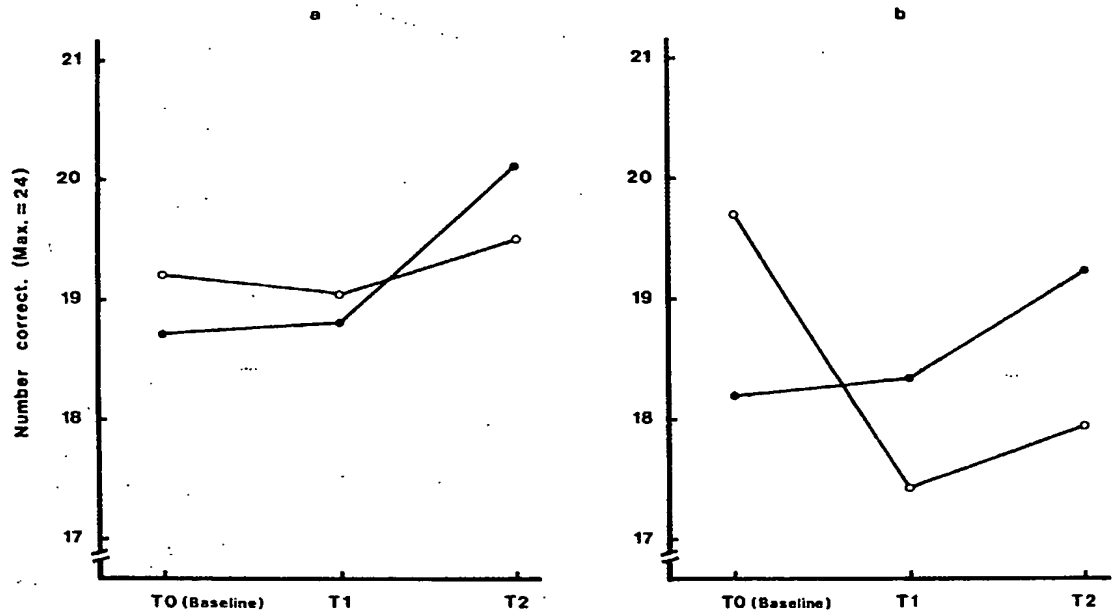

Fig. 3a, b. Correct recognition of abstract shapes with a standard presentation and b concurrent articulation of a 4-digit number. ●—● Placebo; o—o scopolamine (0.6 mg)

responses were slower with concurrent articulation [$F(1,8)=7.06$, $P<0.03$]. This difference was still apparent at T1 [$F(1,8)=5.14$, $P<0.05$], but by T2, subjects were able to respond equally quickly whether they were repeating a number or not. Number of correct responses also decreased significantly with concurrent articulation at T1 [$F(1,8)=57.55$, $P<0.001$], and there were interactions with drug [$F(1,4)=163.8$, $P<0.001$], order of treatment [$F(1,8)=18.33$, $P<0.003$], and with drug and order [$F(1,4)=236$, $P<0.001$]. On tests of simple main effects, there were no differences between conditions and no interactions with order for the placebo group. For the group receiving scopolamine, performance was worse with concurrent articulation [$F(1,8)=80.5$, $P<0.001$], and the decrement was greater for subjects receiving scopolamine in the first session than for subjects receiving it in the second. For T2, there were no indications of differences between presentation conditions at any level.

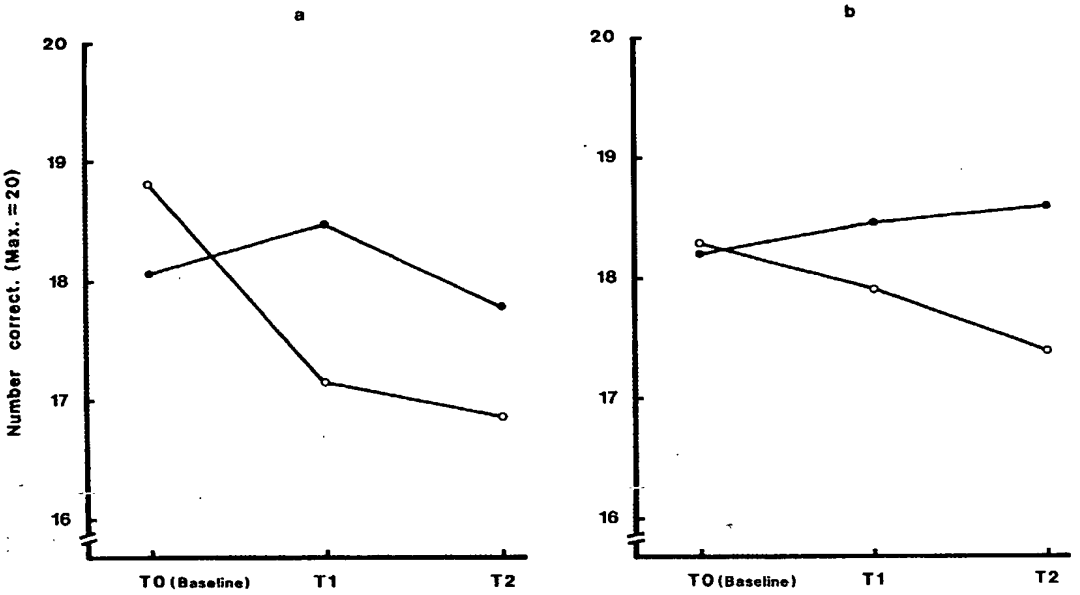

Fig. 4a, b. Memory for spatial location of boxes with a standard presentation and b concurrent articulation of a 4-digit number. ●—● Placebo; o—o scopolamine (0.6 mg)

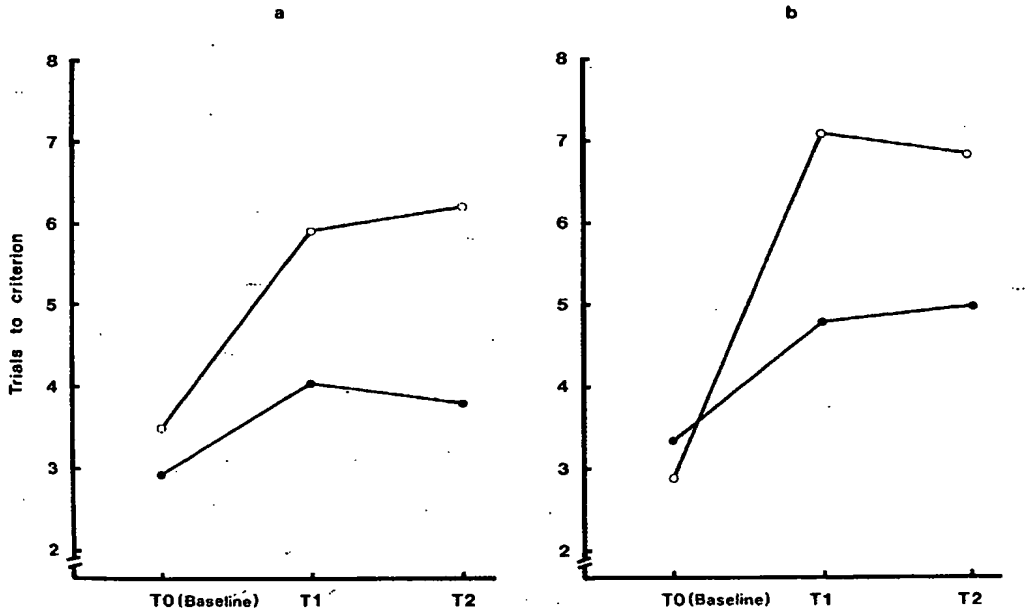

Fig. 5a, b. Number of trials required to learn the correct location of eight abstract shapes (visuo-spatial memory); a standard presentation and b under conditions of concurrent articulation. ●—● Placebo; o—o scopolamine (0.6 mg)

Shape recognition

a. Standard presentation. There were no effects of any of the main factors on recognition memory for shapes, and no interactions (Fig. 3a).

b. With concurrent articulation. There were no effects of drug, order or interactions between these factors at either T1 or T2.

Comparing performance across presentation conditions, concurrent articulation did not impair recognition accuracy

in the pretreatment phase. There was a marginal effect at T1 [$F(1,8)=5.06$, $P<0.06$] and a significant effect at T2 [$F(1,8)=11.17$, $P<0.05$].

Spatial location

a. Standard presentation. The pattern of effects are shown in Fig. 4a. One hour after treatment, memory was significantly impaired by scopolamine [$F(1,18)=14.16$, $P<0.001$]. Performance did not recover under drug, but a decrement in performance under placebo at T2 resulted in a nonsignificant treatment effect.

b. With concurrent articulation. At T1, the placebo group tended to be more accurate than the scopolamine group, and by T2 scopolamine was reliably disrupting performance on this task [$F(1,18)=6.74$, $P<0.02$]; no other effects or interactions were identified.

In an analysis across conditions, there were no main effects of concurrent articulation at any test period. A three-way interaction between order, condition and treatment was significant at T1 [$F(1,8)=7.56$, $P<0.03$]; for standard presentation only, subjects receiving scopolamine in their second session demonstrated a larger drug effect than subjects who received drug in their first session.

Delayed response: visuo-spatial memory

a. Standard presentation. For this task, baseline measures showed differences in the number of errors made on the first trial for the two groups [$F(1,18)=19.6$, $P<0.04$]. At T1, 1 h after treatment, subjects who received scopolamine required more trials to learn the item positions than subjects receiving placebo [$F(1,18)=26.7$, $P<0.001$], and the difference was still significant at T2 [$F(1,18)=20.2$, $P<0.001$]. The increased number of trials reflected the increased number of errors made on trial one [$F(1,18)=19.0$ and 22.0, respectively, for T1 and T2, $P<0.001$] rather than inability to correct errors made. An order × drug interaction on the errors analysis for T2 [$F(1,18)=6.2$, $P<0.03$] indicates a higher proportion of errors by subjects receiving scopolamine in the first session (i.e., the group who had higher error rates in the baseline session also had a higher error rate at T2). The results are shown graphically in Fig. 5a.

b. With concurrent articulation. Baseline performance improved across sessions, independent of treatment [$F(1,18)=5.59$, $P<0.03$].

At T1, there was a main effect of drug, with scopolamine increasing the number of trials required to reach criterion [$F(1,18)=12.10$, $P<0.003$], and the number of errors made on trial one [$F(1,18)=9.04$, $P<0.009$]. The pattern of effects at T2 was exactly the same; main effects of drug on trials to criterion [$F(1,18)=8.19$, $P<0.01$] and errors on trial one [$F(1,18)=10.75$, $P<0.006$] were the only significant effects.

Across presentation conditions, there were no effects of concurrent articulation on performance levels at either baseline or T1. At T2, subjects performing concurrent articulation required significantly more trials to reach criterion [$F(1,8)=5.63$, $P<0.05$]. The emergence of this effect on the final task of the final test period of the session probably reflects interference from concurrent articulation when the subjects are fatigued.

General discussion

The results of the present study provide strong evidence for the disruption of working memory by scopolamine. Under standard test conditions, the problem solving task and tests of visuo-spatial memory showed clear decrements in performance 1 and 2 h after drug administration. There were no significant effects of scopolamine on recognition memory for abstract shapes. The fact that one task on the test battery was unaffected by scopolamine argues against a simple interpretation of the drug effects in terms of an attentional deficit. Further evidence against an attentional hypothesis is provided by the finding that reaction time differences observed in the logic task were unrelated to treatment effects.

The effects of concurrent articulation on task performance suggest that the drug-induced deficits in performance are restricted to specific components of working memory. In the logic task, concurrent articulation interacted with drug treatment. Following scopolamine, performance with concurrent articulation was significantly worse during the first post-drug test phase, but by the second post-drug test phase, effects of concurrent articulation were minimal. Under normal conditions, it is likely that most subjects prefer to use a verbal strategy on this task (as indicated by the general opinion that simultaneous completion of the two tasks was impossible!). However, this must be a preferred strategy rather than an essential one, since the placebo group demonstrated no significant effects of concurrent articulation on this task. Scopolamine apparently impairs the readjustment of strategies, a finding consistent with a drug-induced reduction of central executive resources. Under normal conditions, the availability of the articulatory loop may compensate partly for the impairment of processing resources produced by scopolamine; the drug-induced decrements in performance on the logic task were more marked when this overflow system was disabled.

An interaction between processing load and scopolamine has been suggested by Kopelman (1986), following the report that scopolamine impaired performance on the Brown-Petersen short term memory task (Caine et al. 1981). This task involves a heavier processing load than other short term memory tasks, and is the only one to demonstrate significant impairments with scopolamine. An interaction between processing load and concurrent articulation was noted by Baddeley and Hitch (1974) for reasoning time measures; impaired performance was observed with a 6-digit memory load, but not with either a 2-digit load or articulation of a familiar 6-digit sequence. While Baddeley and Hitch (1974) reported significant effects of load on response time to complete the reasoning task, the drug-induced effect reported here was on number of correct responses. This may reflect a strategic tradeoff, with our subjects placing more emphasis on responding within a given time than with ensuring accurate evaluation. The results of the present study are certainly consistent with the scopolamine-induced disruption of the processing resources of the central executive mechanism, but a stronger argument would have been provided by a demonstration of a drug × difficulty of problem interaction on this task. Unfortunately, the data for this interaction were not collected.

An effect of concurrent articulation was observed only for the shape recognition task in the nonverbal test battery, arguing for verbal mediation in this task. The shapes were

specifically designed to minimise the possibility of effective verbal labelling, but if subjects did provide labels for a few of the items, concurrent articulation would preclude access to the articulatory loop for maintenance rehearsal. The absence of drug effects on this task is consistent with a labelling strategy; scopolamine does not impair digit span (e.g., Mohs and Davis 1985), a task primarily associated with the articulatory loop. It is possible that drug-induced impairments in shape recognition may have emerged with a higher dose of scopolamine. However, 0.6 mg scopolamine administered subcutaneously constitutes a dose in the higher range from human studies, and it seems more probable that the ineffectiveness of the drug treatment was related to processing strategy rather than to dosage.

We have interpreted the effects of scopolamine and of concurrent articulation on problem solving and shape recognition in terms of drug-induced impairments in central executive function. Although the deficits observed in tasks of visuo-spatial and spatial memory may reflect direct effects of scopolamine on the visuo-spatial system, we think this unlikely. Subjective reports of processing strategies in the present study suggest that, for the population tested here, the processing required to complete the visuo-spatial tasks involved central executive resources, either as a supplement to or in preference to the visuo-spatial scratch pad. For example, some subjects reported recoding the spatial positions of the five boxes in terms of relative position or clock positions rather than attempting to image the computer screen with the boxes in situ. The effects of scopolamine in the present study, then, where the tasks do not dictate the strategy or system employed, do not constitute unequivocal evidence of direct effects of cholinergic blockade on the visuo-spatial scratch pad. In a recent study completed in our laboratory (Rusted 1988), we examined the effects of scopolamine on a mental rotation task, where processing is exclusive to the visuo-spatial scratch pad. The drug had no effect on correct performance or response times. We therefore consider that impairments on the visuo-spatial tasks reported here were mediated by central executive involvement.

The scopolamine-induced decrements reported in this study are similar to those reported for Alzheimer patients (Morris 1986; Sahakian et al. 1987), although we observed no drug-induced decrements in the shape recognition test. The cognitive effects of Alzheimer type dementia may prove to be more diverse, and to include damage to subsystem functions, but the results from this drug study present no conclusive evidence of slave system impairments without central executive involvement. We interpret our results as evidence that drug-induced impairments are limited to central executive function. Interestingly, Drachman et al. (1980) argued for a diminished "channel capacity" in the brain following cholinergic blockade, mimicking the pattern of impairments commonly observed with the normal ageing process. The decline in digit span reported in the elderly (Burke and Light 1981) and for Alzheimer patients (Morris 1984) may reflect a failure of the central executive mechanisms to maintain the backup resources necessary to "load" the subsystem efficiently, rather than deficiencies in the subsystems themselves.

Certainly, the additional deficits demonstrated by Alzheimer patients argue for a broader analysis of the neurochemical bases of this disease, but the common pattern of deficits observed in clinical and human experimental models does provide an optimistic note for future research efforts on the cholinergic system.

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Increased cognitive sensitivity to scopolamine with age and a perspective on the scopolamine model

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CONTENTS

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1. INTRODUCTION

Memory impairment is a cardinal symptom of Alzheimer's disease (AD) and is thought to be secondary, at least to some degree, to central cholinergic system pathology[3,17,23,60,71,105,107]. Deterioration of cholinergic system function has also been postulated to contribute to the memory impairment that occurs with normal aging, though the evidence for this is less clear[3,26,54,95]. Studies of the role of the cholinergic system in the memory impairment of AD and of normal aging may help clarify the relationship between the physiological and cognitive changes which occur in these conditions.

The anticholinergic drug scopolamine has been used to model aspects of the memory and other cognitive changes that occur with aging (when administered to young normal volunteers)[5,13,29,37,41] and AD (when administered to older normal volunteers)[88]. In older persons, the impairment produced is consistent with the impression of clinicians that older persons are more sensitive than younger to anticholinergic side effects of drugs such as tricyclic antidepressants, including cognitive impairment[7,24]. Patients with AD have been shown to be more sensitive than aged normals to the cognitive effects of scopolamine in a pharmacologic challenge paradigm, presumably reflecting their cholinergic system deficits[86,87].

The present study was undertaken to conduct a direct comparison of scopolamine's cognitive effects in older as compared with younger subjects, using a consistent dose, route of administration and battery of cognitive tests. A discussion integrating our results with those of prior studies which have used scopolamine to model geriatric memory dysfunction follows.

2. METHODOLOGIC APPROACH FOR SCOPOLAMINE CHALLENGES

2.1. Subjects

Eighteen older (mean age ± S.D. = 66.5 ± 7.9 years) and 46 younger (27.0 ± 6.1 years) normal volunteers participated in the study after informed consent and screening to exclude medical or psychiatric illness. They were paid for their participation and were drug-free for at least 3 weeks prior to the study. Subjects were considered older volunteers if they were at least 50 years old. Demographic data are summarized in Table I.

TABLE I

Demographic data

	Older (n = 18)	*Younger (n = 46)*	*t*	*P*
Age (years)	66.5 ± 7.9	27.0 ± 6.0	21.5	0.0001
Education (years)	17.4 ± 1.7	16.0 ± 2.1	2.5	0.01
WMS [a]	128.1 ± 14.6	113.6 ± 11.5	4.0	0.0002
Weight (kg)	69.4 ± 9.5	72.8 ± 11.0	0.26	n.s.

[a] Wechsler memory scale score. Data are presented as mean ± S.D.

2.2. Experimental design

After completing the battery of cognitive tests at baseline, subjects participated in from two to four study days, each separated by at least 72 h. Subjects were part of one of four different larger studies, each of which included a day when scopolamine alone was administered. Scopolamine, placebo, ($n = 7$) and the other drugs being tested (additional doses of scopolamine, ($n = 7$), thyrotropin-releasing hormone (TRH), ($n = 19$), or amphetamine, ($n = 38$)), were administered in a double-blind, randomized, balanced design across study days. Half-lives of scopolamine, amphetamine and TRH are about 3 h, 4 h and 5 min, respectively. Effects from each of these substances would have abated by the time of the next study day; this was substantiated in the data analysis.

After an overnight fast, an indwelling i.v. catheter was inserted and scopolamine hydrobromide (0.5 mg) was administered over 1 min. The dose of scopolamine was chosen based on a prior study of a range of scopolamine doses which showed that a statistically significant decline in cognitive test performance in older normal volunteers occurred at the 0.5 mg dose[86]. Prior studies have shown that using a fixed dose of scopolamine rather than weight corrected (mg/kg) yields less variable responses to the drug[80]. Cognitive testing was started 90 min after scopolamine was administered, as prior studies have shown that the cognitive effects of i.v. scopolamine peak from 90 to 150 min after administration[80].

2.3. Cognitive tests

Prior to participation in the study, subjects' cognitive performance was documented to be within the range of normal using the Wechsler Memory Scale (WMS)[97] and a battery of tests of learning, memory and attention. This battery of cognitive tests was readministered to subjects on each study day; equivalent forms of the tests were administered in the same order, beginning with the vigilance task, followed by category retrieval and finally the selective-reminding test. In addition, forward digit span was tested prior to scopolamine administration and 120 min after. Descriptions of the tests have been previously published[86] and are summarized below.

2.3.1. Vigilance task. Subjects were read a list of 12 categorically related words, six of which were repeated; subjects were instructed to signal the examiner upon hearing a word for the second time. The number of correctly identified words that had been heard twice (a maximum of six words) was recorded as a measure of vigilance-attention. After a 2-min distractor task, subjects were asked to freely recall items from the list. Subjects were then read a list of 24 categorically related words, 12 of which were from a previously presented list (where six had been presented once and six had been presented twice) and 12 completely new words. Accuracy of the recognition of words that had been presented previously (either once or twice) was recorded as a measure of recognition memory. As words were recognized, subjects were asked to recall how frequently a word had been presented (once or twice). The difference between the mean reported frequency of the once- and twice-presented words was used as measure of automatic memory processes (those which occur without conscious intention)[46,98]. The number of intrusions (subjects' responses that had not been presented from the lists) was recorded during the free recall portion of the vigilance task.

2.3.2. Category retrieval. As a measure of retrieval from knowledge memory, subjects were given two letters (one of three pairs used for the study) and asked to generate words beginning with those letters. They were then given words representing a broad category and asked to generate related words. Responses were recorded over a 90-s period. Stimuli were chosen based on prior data showing that the same mean number of exemplars are generated for each word or letter in normal subjects[4,6].

2.3.3. Selective-reminding task. As a test of episodic learning and memory, subjects were read a list of 12 categorically unrelated words and were asked to recall them; missed words were repeated by the examiner and recall of the entire list was again attempted, for a total of eight trials. A free recall and a consistency of recall score were recorded[10]. This task is also thought to provide a measure of effort-demanding cognitive

TABLE II

Cognitive test performance in younger (n = 46) as compared with older (n = 18) normal volunteers

Data are presented as the mean ± standard deviation. Parentheses enclose the range of possible scores for tests

Cognitive test	*Drug*	*Old*	*Young*	*F*
Vigilance-attention (0–6)	baseline	5.7 ± 0.6	5.4 ± 0.9	
	scopolamine	4.5 ± 2.2	5.3 ± 0.8	4.1 †
Free recall				
Once-presented (0–6)	baseline	3.9 ± 1.6	3.3 ± 1.4	
	scopolamine	2.2 ± 1.5	2.6 ± 1.5	3.2
Twice-presented (0–6)	baseline	4.8 ± 0.8	5.2 ± 0.8	
	scopolamine	2.5 ± 1.7	3.9 ± 1.2	4.7 *
Recognition				
Once-presented (0–6)	baseline	5.2 ± 0.9	5.4 ± 0.9	
	scopolamine	4.5 ± 1.5	5.0 ± 0.8	0.3
Twice-presented (0–6)	baseline	6.0 ± 0.0	5.9 ± 0.2	
	scopolamine	5.2 ± 1.5	5.7 ± 0.6	1.6
Word frequency (0–1.0)	baseline	0.4 ± 0.2	0.5 ± 0.3	
	scopolamine	0.3 ± 0.3	0.4 ± 0.3	0.7
Category retrieval				
Words [a]	baseline	26.0 ± 13.9	26.5 ± 7.9	
	scopolamine	18.1 ± 9.1	21.5 ± 6.4	1.9
Letters	baseline	24.3 ± 6.9	31.3 ± 13.7	
	scopolamine	16.3 ± 8.8	31.8 ± 17.7	12.8 **
Selective reminding				
Free recall (0–12)	baseline	8.4 ± 2.2	10.0 ± 1.5	
	scopolamine	4.7 ± 2.8	7.7 ± 2.0	6.4 *
Recall consistency (0–1.0)	baseline	0.9 ± 0.1	0.9 ± 0.2	
	scopolamine	0.4 ± 0.2	0.6 ± 0.2	17.9 **
Forward digit span	baseline	6.3 ± 1.2	7.8 ± 1.1	
	scopolamine	5.5 ± 1.5	6.8 ± 1.2	0.6
Intrusions	baseline	0.5 ± 0.6	0.9 ± 1.0	
	scopolamine	1.4 ± 2.2	0.8 ± 1.0	3.7 †

* $P < 0.05$, ** $P < 0.001$, † $P < 0.06$ for age × drug interaction.

[a] For this task data were available for 12 older and 25 younger subjects.

processes, or those that require sustained attention and effort[46,98].

2.3.4. Digit span. Subjects were read random sequences of digits and were asked to recall them immediately in the sequence given as a measure of immediate memory and attention[96].

2.4. Behavioral measures

A physician-investigator completed rating scales for 12 of the older subjects and a cognitive tester completed them for 32 of the younger subjects, under blind conditions, to evaluate behavioral effects of scopolamine prior to drug administration and at the completion of cognitive testing on each study day. 100 mm visual analog scales (VAS) were used[65,86,87,89], with zero being the low score, for the items 'alert', 'fatigued/tired', 'trouble concentrating', 'sad/depressed' and 'anxious'. Ratings were based on staff observation and direct questioning of subjects on each item.

2.5 Methodologic issues

The study was designed to compare the cognitive effects of 0.5 mg of i.v. scopolamine in older as compared with younger normal volunteers. It is well established from numerous prior studies that the cognitive effects of scopolamine differ from placebo[5,9,13,20,29,37,41,53,86–88,103]. In addition, several studies using paradigms similar to that in the present study established that cognitive test performance at baseline, prior to a subject's participation in drug studies, is not significantly different from cognitive performance after administration of a placebo[83,86,90,103]. Retrospective ANOVA of data from another study,[86] which included ten older normal volunteers, showed no differences between baseline and placebo day cognitive performance on all tests except word retrieval from specified categories (unpublished data available on request). For these reasons and the fact that the side effects of scopolamine are such that even when a placebo control is used, the blind is compromised,[86,92] a true placebo day was not included for most of the subjects in the present study.

2.6. Statistical analysis

The clinical characteristics of the two age groups were compared using Student's *t*-tests. Cognitive variables were examined using repeated measures design ANOVA comparing the mean scores of the two groups at baseline and following scopolamine treatment. Of the relationships tested in these analyses, the age group × drug interaction was of primary interest because it indicated whether a differential, age-related response to drug treatment occurred (Table II). To further elucidate this interaction, baseline versus scopolamine scores within each age group were compared using post hoc Bonferroni-corrected *t*-tests (Table III). One-tailed probabilities were used for these tests, because of the numerous studies showing a decline in cognitive performance after scopolamine administration. To assess the effects of gender, weight,

TABLE III

Within age group changes in cognitive test scores after scopolamine, expressed as the difference between baseline score and score after scopolamine in younger (n = 18) and older (n = 46) normal volunteers

Data are presented as the mean ± S.D. and were analyzed using post-hoc *t*-tests.

	Older	*t*	*Younger*	*t*
Vigilance attention	−1.2 ± 2.3	−2.3 *	−0.1 ± 1.1	−0.5
Free recall				
Once presented	−1.7 ± 1.8	−4.0 **	−0.7 ± 2.2	−2.3 *
Twice presented	−2.3 ± 1.6	−6.2 **	−1.4 ± 1.5	−6.2 **
Recognition				
Once presented	−0.7 ± 1.9	−1.4	−0.4 ± 1.0	−2.5 *
Twice presented	−0.8 ± 6.2	−5.5 *	−0.3 ± 0.7	−2.9 *
Word frequency	−0.1 ± 0.4	−1.4	−0.2 ± 0.4	−0.7
Category retrieval				
Words [a]	−8.0 ± 10.7	−2.6 *	−5.0 ± 8.1	−3.1 *
Letters	−8.0 ± 6.2	−5.5 **	0.6 ± 9.3	0.4
Selective reminding				
Free recall	−3.7 ± 2.3	−6.8 **	−2.3 ± 1.8	−8.9 **
Recall consistency	−0.5 ± 0.2	−9.1 **	−0.3 ± 0.2	−8.3 **
Forward digit span	−0.8 ± 0.9	−4.7 **	−1.1 ± 1.4 *	−5.4 **
Intrusions	0.9 ± 2.1	1.9 †	−0.1 ± 1.4	−0.3

** $P < 0.001$, * $P < 0.05$, † $P < 0.1$ for the difference between baseline score and score after scopolamine.

[a] For this task, data were available for 25 younger and 12 older subjects.

education level and general level of cognitive performance (as measured by the WMS) the ANOVAs were repeated using these factors as covariates. In addition, to determine whether there were carryover effects between the treatment conditions, the original ANOVAs were first performed with the order of drug treatment included in the analysis model; since order was not found to be a significant variable, it was dropped from further analysis. Further explorations of the relationship between age and cognitive test scores were made using Pearson's product-moment correlations. ANOVA was used to detect time and, therefore, drug effects on the VAS scale measures. All data are presented as the mean ± 1 standard deviation.

3. RESULTS OF SCOPOLAMINE CHALLENGE IN DIFFERENT AGE GROUPS

3.1. Cognitive tests

The mean scores for each test during baseline and scopolamine conditions are summarized in Table II. All subjects completed cognitive testing and none became delirious. Scores on baseline cognitive tests were significantly lower in the group of older as compared with younger subjects on the free recall portion of the selective-reminding test, letter fluency and digit span. The drug × age interaction arising from the ANOVA for each test indicated that the older subjects were significantly more sensitive to the cognitive effects of scopolamine on the selective-reminding test, letter fluency and free recall of twice-presented words (Table II, Fig. 1). Regarding the latter task, the older subjects were able to utilize the repeated presentation of a word for recall at baseline, since recall of twice-presented items was significantly greater than that of once-presented words ($t = 2.3$, df = 17, $P < 0.04$). This priming advantage was greater in the younger group at a trend level of significance ($t = 1.9$, df = 45, $P < 0.06$). After receiving scopolamine however, the repetition advantage seen at baseline was lost in the older subjects, as there was no significant difference between their recall of words presented once and twice ($t = 0.7$, df = 17, $P < 0.52$), though the improved recall after repetition, even after scopolamine, was retained in the younger subjects ($t = 5.1$, df = 45, $P < 0.001$).

The ANOVA demonstrated that scopolamine caused a significant decline in performance on all cognitive tests with the exception of word frequency and intrusions. For comparison purposes and following from the ANOVA, drug effects as evaluated using post hoc *t*-tests done within each age group are summarized in Table III.

In addition, older subjects were more sensitive than younger at trend levels of statistical significance on the vigilance-attention measure and on number of intrusions produced (Table II). Letter fluency, vigilance-attention score and intrusions were not affected by scopolamine in the younger group, though these tests were affected in the older subjects, the latter one at a trend level of significance (Table III).

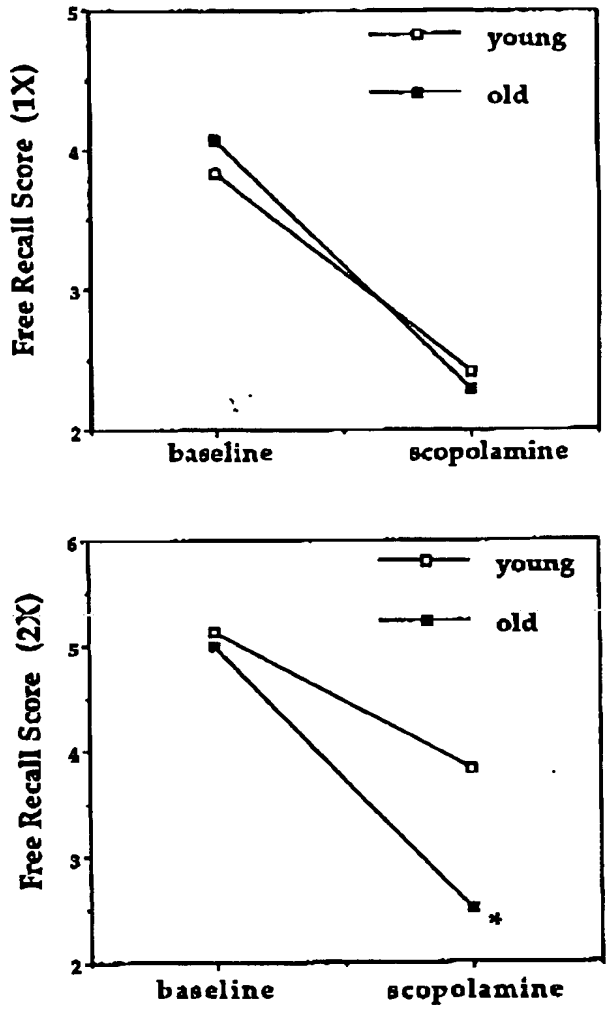

Fig. 1. Older subjects were not able to use the repetition of a word as an aid to encoding as the younger subjects could after scopolamine ($F_{2,62} = 5.5$, $P < 0.02$). Shown are the mean baseline and post-scopolamine scores for the younger and older groups for the free recall of once- and twice-presented words from the vigilance task.

Correlations between age and delta scores were significant for selective-reminding test free recall ($r = -0.38$, $P < 0.04$) and consistency ($r = -0.56$, $P < 0.0001$) (Fig. 2), vigilance-attention ($r = -0.34$, $P < 0.01$), free recall of twice-presented words ($r = -0.27$, $P < 0.04$), letter fluency ($r = -0.51$, $P < 0.0001$) and number of intrusions ($r = 0.26$, $P < 0.05$). Neither gender, weight, nor education level as covariates significantly altered the drug × age effects seen in the cogni-

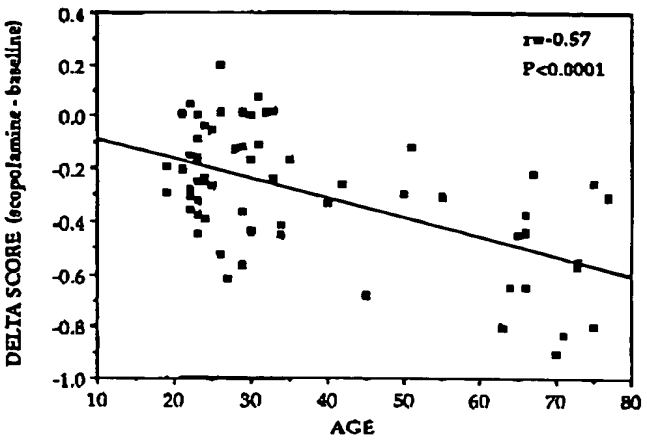

Fig. 2. Age correlates inversely with the selective-reminding test consistency delta score ($n = 64$).

tive test scores. Drug × age effects were enhanced using the WMS score as a covariate for the number of intrusions produced; no effect on the other cognitive tests was observed.

3.2. Behavioral measures

Data from the VAS scales administered prior to scopolamine administration and immediately after cognitive testing showed that the measure 'alert' was significantly decreased ($F_{1,41} = 44.1$, $P < 0.0001$) and 'fatigued/tired' and 'trouble concentating' were significantly increased ($F_{1,41} = 41.0$, $P < 0.0001$ and $F_{1,41} = 21.1$, $P < 0.0001$, respectively) by scopolamine in the overall group of subjects. 'Sad/depressed' and 'anxious' were not significantly affected. About half of the cognitive test scores of each age group correlated significantly with the behavioral measures of alertness and fatigue, with correlation coefficients ranging from 0.54 to 0.70. Therefore, even for the tests in which a significant correlation was present, the largest part of the variance was apparently still due to other factors. The study was not originally designed for comparison of the behavioral measures between age groups and the difference in clinical training and experience between the raters for the two age groups unfortunately precluded such a comparison.

4. DISCUSSION OF SCOPOLAMINE EFFECTS BETWEEN AGE GROUPS

4.1. Increased sensitivity to scopolamine-induced cognitive impairment with age

Two main interelated points are raised by our results. First, older people appear to be quantitatively more sensitive to scopolamine than younger people on some tests of episodic learning and memory and qualitatively more sensitive (older subjects' scores declined significantly while younger subjects' did not) on a letter fluency test and possibly on a vigilance-attention test and the production of intrusions. This increased sensitivity to cholinergic blockade may indicate that the memory impairment that occurs as a result of aging is due, at least in part, to decreased cholinergic system function. Second, the scopolamine model of the cognitive impairment of dementia has been used in many studies of both animals and humans; our results indicate that age is an important variable to consider in the use of this model.

Our results are consistent with data from three other studies which examined scopolamine's cognitive effects in younger and older people[36,73,109]. Those studies found increased scopolamine sensitivity in the elderly on tests of recent memory and constructional praxis.

4.2. Aging and the cholinergic system

Evidence from neuropathological and biological studies has been conflicting on whether cholinergic system deficits in the elderly are of sufficient magnitude to produce cognitive impairment[3,26,105]. The increased anticholinergic sensitivity demonstrated in older subjects in this study is consistent with cholinergic system decline with age, as patients with AD who have well documented cholinergic system pathology,[3,17,23,107] show an increased sensitivity to scopolamine as compared with normal older controls[86].

Deficits in several aspects of the cholinergic system have been documented to occur with age. The changes that have been found most consistently are decreased acetylcholine (ACh) synthesis,[26,42] decreased ACh release[26,108], and decreased responsivity of cholinergic neurons to ACh[3,26,57]. Several studies have also documented decreases in the numbers of brain muscarinic receptors with age, in both animals[70,82,95] and humans.[67,75,104].

Drugs that enhance cholinergic neurotransmission have been shown to enhance cognitive performance of aged monkeys and other animals[3,8] and aged humans[25,30]. A few studies have shown a more direct relationship between cholinergic system changes and cognitive performance. One animal study reported a correlation between decreased choline acetyltransferase level in the hippocampus and performance on a maze-running task[47]. In a study of cortical and hippocampal tissue of young and aged mice after completion of a memory task, a significant increase in high affinity choline uptake, implicating activation of cholinergic ascending neurons, was found in young, but not old animals[27,54].

4.3. Possible confounding factors of age-related anticholinergic sensitivity

Additional reasons older subjects may be more sensitive to the cognitive effects of scopolamine include an increased sensitivity to the sedative effects, or possibly decreased metabolism and excretion of the drug, resulting in relatively higher blood levels, as can occur with other drugs with age[44]. The contribution of these factors cannot be determined from the data from the present study, though the fact that all subjects completed cognitive testing and that the impairment of the older as compared with the younger subjects' performance was significantly different on only some of the tests supports a true differential effect on memory. In addition, the difference between baseline and performance after scopolamine on the measures of attention used, the vigilance-attention score and digit span, were not significantly different between the two age groups (Table II). Baseline cognitive test performance as measured by the WMS and the mean educational level were higher for the older as compared with the younger subjects (Table I), so older subjects were actually advantaged in these regards.

In one prior study, serum anticholinergic activity was not significantly different between groups of older and younger subjects after three different doses of scopolamine[73]. In addition, two other reports support that a pharmacokinetic difference does not explain the increased cognitive sensitivity to scopolamine in the elderly, in that other anticholinergic side effects were comparable between age groups[36,109].

4.4. Comparisons between cognitive processes in aging and after scopolamine

Our results are generally in agreement with the original study by Drachman and Leavitt and others who have shown that scopolamine produces a pattern of cognitive impairment in young normal volunteers that is similar to the cognitive impairment which occurs in aging[29,37]. Cognitive decline has been most consistently demonstrated in normal aging on tests involving new learning and episodic memory, psychomotor speed, concept formation, visuospatial praxis and attention[11,35]. Areas of cognitive functioning that are generally considered to be preserved with age are immediate memory, language and retrieval from knowledge memory[35].

Scopolamine had a relatively greater effect in the older subjects on tests in which repetition of to-be-remembered items was involved; this was statistically significant on the selective-reminding test and free recall of twice-presented words (Table II; Fig. 1). Repetition of a stimulus word provides additional opportunities to encode information that may already be stored in memory. Older subjects, after scopolamine treatment, were not able to utilize and benefit from the second presentation of an item as the younger people could. In the encoding of information into memory, older people do not utilize cues, such as the second presentation of a word, as well as younger people[18]. The increased sensitivity of older people to the effects of scopolamine on such tests is consistent with the involvement of the cholinergic system in the encoding of information into memory and a decline in that system with age. The repetition advantage, or priming effect has been noted to be affected by scopolamine in prior studies[21,66].

Retrieval from knowledge memory has not been shown to decline with age[29,35], though in the present study, scopolamine caused a significantly decreased category retrieval score in both younger and older subjects (Table III). Possibly, cognitive processes utilized for this test other than retrieval from knowledge memory are affected with age. A decline in frontal lobe function for example, which has been shown to occur in some older people[18] may affect performance on this timed task by increasing the time to initiation of list generation and impairing ability to continue to generate responses over time.

Digit span is not affected by aging[29,35], though it decreased after scopolamine in both age groups in the present study (Table III). This is consistent with findings from prior studies, which have shown an effect of scopolamine on digit span when higher doses, more direct routes of administration, or older subjects were used[37,66,86]. In other studies, digit span was not affected[9,29,36,41,53,80,109].

4.5. Comparisons between cognitive processes in Alzheimer's disease and after scopolamine

In elderly normals, scopolamine has been reported to produce deficits in cognitive test performance not unlike some of those seen in patients with AD[36,73,86,88,109]. Our results are consistent with those findings, in that scopolamine affected the performance of the older subjects on tests of episodic learning and memory, retrieval from knowledge memory, vigilance-attention, digit span and production of intrusions. They are inconsistent in that no effect was found on estimation of word frequency, which was used as a measure of automatic processing, which has been shown to decrease in AD[43].

The older subjects were more impaired than the younger on recognition and recall of twice-presented words (Fig. 1) and also on the selective-reminding test, which involves the repetition and recall of a word list

(Table II, Fig. 2). This aspect of the scopolamine-induced impairment in the older subjects then, is similar to test performance in patients with AD, who have been shown to be unable to use repetition as an aid to encoding into memory[81,85].

The increased number of intrusions errors in the older subjects after scopolamine is consistent with results from some[9,29] though not all prior studies[5,36,37,61,91]. As an index of memory impairment, a relatively greater increase in intrusions in the older subjects is consistent with their poorer performance on recall tasks. One group of investigators demonstrated a dose-dependent increase in intrusions after scopolamine[9]. All previous studies examining this measure tested subjects that were considerably younger than the older subjects in the present study. Intrusion errors are of interest because studies have shown that they are very characteristic of AD patients, even to the extent of being an aid to the differential diagnosis of dementia[5,39]. Intrusion errors have also been associated with low cortical choline acetyltransferase activity and with senile plaques in AD patients[39,84].

Automatic processing (memory that occurs without conscious intention) is impaired in patients with AD[43] and was affected by scopolamine in one prior study using recall for temporal context as the task[74]. In the present study, using estimation of word frequency as the task, no significant effect was detected in either age group.

Patients with AD have visuospatial deficits, including visuospatial memory[48,64]. Several studies have shown that scopolamine produces visuospatial memory impairment,[13,29,37,53-55,72], though two studies found no impairment[9,79]. Impairment on tests of visuospatial praxis (block construction and digit symbol) also has been demonstrated[29,36,37,109].

5. LIMITATIONS OF THE SCOPOLAMINE MODEL

The scopolamine model has been used extensively to learn more about the status of the cholinergic system and its role in cognition in aging and AD and in the evaluation of possible therapeutic agents for memory impairment[3,5,9,13,20,29,37,53,61-63,72,83,86,88,100,101]. Variable results have been reported from many of these studies, which are probably secondary in large part to the various routes of scopolamine administration, doses and cognitive test batteries used. Different methods of administration affect drug levels and the time to peak effects; i.v. administration, for example, has been shown to be 42% more centrally potent than intramuscular[49]. Subcutaneous administration produces slower absorption[76], and the oral/i.v. effective dose is about 4:1[80]. Limitations of the use of scopolamine's effects as a model arise from discrepancies between its effects and the cognitive impairment which occurs in AD. These include the lack of an effect of scopolamine in most studies on measures of immediate memory, retrieval from knowledge memory, naming and other language function and retrograde memory[5,9,20,31,36,37,45,53,56,72,91,101]. Some of these and other problems are discussed below.

5.1. Scopolamine's effects on sedation and attention

A frequent criticism and problem associated with the evaluation of the cognitive effects of scopolamine is that it causes sedation, which may interfere with alertness, attention and vigilance and contribute to apparent memory impairment[9,14,15,20,32,33,72,101]. Arguments against the idea that sedation is a major contributor to the memory-impairing effects of scopolamine come from studies which have shown that its effects on measures of vigilance and attention, or sedation are separate from effects on memory impairment[3,13,19,20,29,52,53,73]. In addition, lorazepam, at doses that produce sedation comparable to that produced by scopolamine does not produce cognitive impairment[89]. Furthermore, most, though not all attempts[61] to reverse scopolamine induced cognitive impairment with stimulant drugs have been unsuccessful[2,28], while cholinergic drugs consistently do[2,28,61,72,83]. We recently confirmed the former using dextroamphetamine (using both 0.25 and 0.5 mg/kg administered orally), using the same cognitive tests as in the present report and the same dose of scopolamine (Martinez et al., submitted). Interestingly, the fact that recall of words presented twice as compared with those presented only once, at least in the young people after scopolamine, argues for an effect on memory, in that subjects must have been able to attend adequately in order for them to take advantage of the repeated presentation of the stimulus words for encoding. Nevertheless, some authors believe that interference with attention or early processing of information has much to do with the memory deficits produced by scopolamine[15,32,33,45].

The concepts of attention and vigilance, their relationship to memory test performance and their measurement are difficult and controversial[9,32]; more work needs to be done in the area of early information processing under both normal and drug conditions. From the available information, the effects of scopolamine on tests designed to measure attention, vigilance and alertness are task and dose dependent. For example, as discussed by prior investigators, tasks with high information loads are more likely to be sensitive to effects of pharmacologic interventions and have

been consistently found to be impaired after scopolamine treatment[52,101]. In general, reaction time, stimulus discrimination, digit span and evoked potential are impaired at higher doses and measures of sustained attention are impaired at lower doses[14,72,80,94,102]. For more detailed reviews of studies of early information processing after scopolamine, see[9,33,52,101]. In terms of modelling, an attentional deficit would be consistent with AD impairment[64,68,69] and with the known involvment of the cholinergic system in attentional mechanisms[14,29,32,93,94].

As a corollary to prior work examining attentional systems after scopolamine, the model of working memory as put forth by Baddeley[1], in which a system termed the central executive is concerned with allocating attentional resources, was used to study scopolamine's effects[78,79]. Working memory in the model involves both the storage and processing of information. The central executive component of working memory, as evaluated using free recall and problem solving tasks concurrent with performance of secondary tasks which compete for the same processing facilities, was found to be impaired after scopolamine. Other components of this working memory system, the phonological loop and the visuospatial scratchpad were not affected by scopolamine[79]. These authors also argued that because scopolamine did not effect all tests examined that the effects of the drug were secondary to more than just an attentional deficit.

5.2. Scopolamine's effects on implicit memory

A number of studies have examined the effects of scopolamine on measures of implicit memory such as priming or procedural memory. In these types of tasks memory is expressed implicitly by facilitation of performance secondary to prior experience with the task[66]. Two studies found that scopolamine did not significantly effect performance on skill learning tasks[53,66], though others found significant impairment using a pursuit rotor task[20] and a tracking task[38]. Another study found that scopolamine impaired performance on a verbal serial reaction time task[50].

Scopolamine decreased the repetition priming effect after a 60-min though not a 5-min delay, using a word fragment completion test[66]; another study using a similar test also suggested a role for the cholinergic system in priming effects[21]. These studies are consistent with impaired priming demonstrated in the older subjects from the present study. Others researchers found no effect on repetition priming or word stem completion[50,53]. Only one study to date though has attempted to control for explicit memory contamination of performance on the implicit tasks. In that study, diazepam was administered as a control drug; it caused a decrease in explicit recall, with implicit memory intact. This contrasted with performance after scopolamine, in which performance on explicit and implicit tasks correlated[21]. Measures of procedural memory and priming are certainly impaired in patients with AD,[81,85] though interestingly some studies of early AD patients have shown procedural memory function to be relatively spared[34,51]. Our results and those of prior studies indicate that there may be a continuum of effects of scopolamine on implicit memory functions, with important variables being subjects' age, dose and tasks used[21,38,50,53,66].

5.3. Scopolamine's effects on retrieval from knowledge memory

The effects of scopolamine have been inconsistent on measures of retrieval from knowledge memory among different studies. The lack of an effect on such measures has been one of the primary arguments against the scopolamine model of dementia[5,20,31,45,53,56,72,92], as patients with AD are substantially impaired in retrieval from knowledge memory[12,92,99]. Studies which have found knowledge memory impairment after scopolamine used category retrieval tasks, relatively high doses and/or older subjects as in the present study[13,29,65,86,91]. Two of these studies clearly showed the dose-dependency of the effect[65,86]. Troster and coworkers reported an impairment in category retrieval in young subjects after scopolamine, but not on other tests designed to evaluate retrieval from knowledge memory[91]. These tests included photograph identification of famous people from past years, recall of famous past events and geographical knowledge. As those authors pointed out, more than one measure of retrieval from knowledge memory should be used.

5.4. Other limitations of the scopolamine model

Any model, especially of something as complicated as memory impairment, will have limitations. It is unrealistic to think that transient deficits secondary to acute drug challenge will precisely model changes in cognitive functioning that occur over a long period of time. Further complicating matters, neurotransmitter deficits other than those in the cholinergic system occur in AD and probably contribute to the memory and other cognitive impairments in the disease[3,22,58,77,106,111]. In general, the cholinergic system interacts with other neurochemical systems and some of these have been shown to decline in normal aging[16,40,54]. Th noradrenergic system has been the most studied in this regard; its interactions with the

cholinergic system in cognitive operations is just beginning to be defined and understood[54,59,110].

6. SUMMARY AND CONCLUSIONS

18 older normal volunteers (mean age = 66.5 ± 7.9 years) and 46 younger volunteers (mean age = 27.0 ± 6.1 years) were administered the anticholinergic drug scopolamine (0.5 mg i.v.) followed by a battery of cognitive tests evaluating attention, learning and memory. The older subjects were significantly more impaired than the younger by scopolamine on some tests of learning and memory. This increased sensitivity of the older group to scopolamine is consistent with studies in animals and humans showing decreased cholinergic system function with age. The findings also indicate that age is an important variable to consider in using the scopolamine model of memory impairment. The cognitive impairment caused by scopolamine in younger subjects in this and prior studies is similar to some, but not all aspects of the impairment which occurs in normal aging[11,29,37]. Scopolamine also caused impairments on digit span and word fluency tasks, which are not consistent with normal aging changes. In the older group of subjects, scopolamine produced aspects of the cognitive impairment which occurs in AD on tests of episodic memory and learning, vigilance-attention, category retrieval, digit span, and number of intrusions[64,86,88,91]. Other areas of cognition that are of relevence to aging and AD such as psychomotor speed, praxis, concept formation and remote memory were not evaluated in this study. Some of these are being evaluated in ongoing studies, along with additional and more specific tests of retrieval from knowledge memory, implicit memory and attention. The scopolamine model has provided a fruitful pharmacologic starting point for the study of a number of cognitive operations. The idea of dissecting apart aspects of memory systems pharmacologically depends on the availability of neurochemically specific drugs and on the specificity and sensitivity of neuropsychological tests for distinct cognitive operations or domains. Further studies using such tools will aid not only in the understanding of the impairments which occur in aging and in AD, but also of the conceptualization of memory and other cognitive operations and ultimately the physiological mechanisms involved in memory and learning.

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IN THE MATTER OF
US Serial No: 09/147,490
entitled"Neuroactive Peptide"

EXHIBIT 7

This is Exhibit 7 referred to in Clause 14of the Statutory Declaration Siew Yeen Chai dated 13th Day of January 2004.

Before me: _____

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A registered Patent Attorney within the meaning of the Patents Act 1990.

Indian Journal of Pharmac logy 2003; 35: 104-108 SHORT COMMUNICATION

EFFECT OF METOCLOPRAMIDE ON SCOPOLAMINE-INDUCED WORKING MEMORY IMPAIRMENT IN RATS

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ABSTRACT

Objective: To evaluate the effect of metoclopramide on scopolamine induced working memory deficits in rats by using the three-panel runway apparatus.

Methods: Male albino rats trained to reach the goal box were randomly divided into groups of 6 animals each. The groups were administered intraperitoneaiiy saiine, scopolamine (0.1-0.56 mg/kg) and metoclopramide (0.1-5.0 mg/kg). The working memory errors and latency period of the session were recorded on the three-panel runway apparatus.

Results: Treatment with scopolamine (0.1 mg/kg) did not result in any significant change in working memory errors or latency period while in a dose of 0.56 mg/kg resulted in a significant increase in the number of working memory errors and latency period. Concurrent administration of metoclopramide (1.0 mg/kg) and scopolamine (0.56 mg/kg) resulted in a significant increase in working memory errors as compared to the scopolamine alone. Rats co-administered with metoclopramide (5.0 mg/kg) and scopolamine (0.56 mg/kg) failed to complete the trial run.

Conclusion: We conclude that scopolamine treatment resulted in working memory deficits on the three-panel runway apparatus. Metoclopramide (1.0 mg/kg) significantly aggravated the number of working memory errors produced by scopolamine. Metoclopramide was without any effect on the latency period.

KEY WORDS Dopamine antagonist memory deficit runway apparatus

INTRODUCTION

Cognitive impairment is seen in a wide variety of neurodegenerative disorders[1]. Working memory is a form of short-term memory with a limited capacity and an extremely rapid decay[2]. Its impairment is more depictive of memory disorder in the Alzheimer's dementia[2]. Although multiple neurotransmitters are involved in cognitive deficits, the most striking and consistent change is observed in the central cholinergic system[3]. Scopolamine, a muscarinic receptor antagonist, induces amnesia in various animal models and is a widely cited model for human dementia. Scopolamine has been reported to impair passive avoidance task[1], spatial memory deficits[4], and working memory impairment in radial arm maze[5] and in the three-panel runway apparatus[6,7].

A growing number of reports have indicated a role for 5-HT_3 and 5-HT_4 receptors in cognition. Considerable evidence indicates that 5-HT_3 receptor antagonism and 5-HT_4 receptor-mediated activation in hippocampal neurons, facilitates the induction of long-term potentiation (LTP), which is regarded as a cellular basis of memory[8]. Antagonists at 5-HT_3 and agonists at 5-HT_4 receptors facilitate cognitive performance in various animal studies, possibly through acetylcholine release[9]. Antagonists at 5-HT_3 receptors have demonstrated to reverse scopolamine-induced impaired task in passive avoidance

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paradigm[10] and Morris water maze[11]. Several agents exhibiting 5-HT_4 receptor agonist activity facilitate cognitive performance in animal models[12].

Metoclopramide, a benzamide derivative exhibits diverse receptor properties. Metoclopramide is a 5-HT_3 antagonist, weak 5-HT_4 agonist and dopamine receptor (D2) antagonist. Metoclopramide prevented dicyclomine induced amnesia in the passive avoidance test[13]. Furthermore D2 receptor antagonists were reported to cause disruption of passive avoidance task[14] and impaired working memory performance[15]. Because of multiple receptor actions, the effect of metoclopramide on cognitive functions is difficult to predict. We therefore investigated the effect of metoclopramide on scopolamine-induced working memory deficits on the three-panel runway apparatus in rats.

MATERIALS AND METHODS

Animals: Male albino rats of Wistar strain were used in the three-panel runway task. Initially their free feeding weights were 230-290 gm. Their body weights were maintained at approximately 80% of the free feeding level during the experimental period. The rats were provided commercial food pellet and water *ad libitum*. Animals were housed in groups of 3-4 per cage and kept under controlled room temperature (24±2°C) in a 12 h light-dark cycle. All experiments were conducted between 0900 and 1700 h in a noise free environment. The institutional ethical committee had approved the study.

Apparatus: Working memory was assessed with a three-panel runway apparatus[6]. In brief; this apparatus has a start box, a goal box and 4 consecutive intervening choice points. Each choice point consisted of 3 panels or gates. The rats were prevented from passing through two of the three panels or gates, by front stoppers and also prevented from returning to the start box or to the previous choice point, by the one-way opening hinged panel gate. When the rats reached the goal box, they received two food pellets, of about 50 mg each.

Acquisition training: Initially all the front stoppers were removed so that a rat could pass through any of the 3 panel gates at each choice point. The rats were made to run the task repeatedly until the time that elapsed from leaving the start box to reaching the goal box (latency period) was consistently less than 30 sec. Once this time was reached, the rats were given 1 session of 6 consecutive trials per day, with inter-trial period of 2 min. Each day, the sequence of correct panel gate position (open gate) for each rat, was changed according to the sequence chart[6]. The number of times an animal pushed an incorrect panel-gate (errors) and the time required for the animal to reach the goal box (latency period) were recorded in every trial of the session. Errors and latency periods of each of the 6 trials were added to obtain the total number of errors and total latency period of the session. A rat was selected for the experiment if it achieved the criterion of ≤12 mean errors per session in 3 consecutive sessions.

Drugs: Scopolamine [Sigma Laboratories, USA] was administered in three doses of 0.1, 0.32 and 0.56 mg/kg, 20 min before the runway session. Hospital supply of metoclopramide ampoules were diluted in distilled water and was administered 20 min before the runway task, in the doses of 0.1, 1.0 and 5.0 mg/kg. All the drugs and saline were administered intraperitoneally (*i.p.*) at a volume of 0.1 ml per 100 gm body weight. Drug or saline treated trained rats, if failed to reach the goal box within the cut off time period of 2 min, were not included in the results.

Data analysis: Working memory errors and latency period per trial and session are presented separately and expressed as mean±SEM. The comparison of difference in errors and latency periods between different groups was determined with a one-way ANOVA, followed by Dunnett's multiple comparisons test when F-ratios reached significance ($P<0.05$).

RESULTS

Acquisition training: In the three-panel runway task, the random performance level was four errors per trial and 24 errors per session. Rats were trained in around 15 sessions. With repetition of training, the number of errors made from the second to sixth trial (working memory errors) decreased markedly while errors observed in the first trial remained constant at approximately four (Table 1).

Working memory errors: Scopolamine treatment in the dose of 0.1- 0.56 mg/kg resulted in a dose dependent increase in working memory errors, an effect that reached significance for the dose of

Table 1. Effect of scopolamine and metoclopramide on working memory errors in albino rats on three-panel runway apparatus.

Group@	Numbers of errors in each trial						Total
	1	2	3	4	5	6	
Saline (control)	3.83±0.40	1.83±0.31	1.00±0.45	0.67±0.33	0.33±0.21	0.17±0.17	7.83±0.49
Scopolamine (0.1 mg/kg)	3.83±0.31	2.83±0.30	1.67±0.21	1.00±0.37	0.83±0.31	0.67±0.21	10.83±0.79
Scopolamine (0.32 mg/kg)	4.17±0.31	3.17±0.40	2.00±0.45	1.67±0.33	0.67±0.33	0.67±0.21	12.35±1.52
Scopolamine (0.56 mg/kg)	4.50±0.34	3.17±0.48	2.00±0.26	2.17±0.31	1.83±0.31	1.83±0.31	15.50±1.41*
Metoclopramide (0.1 mg/kg) + scopolamine (0.56 mg/kg)	4.33±0.21	3.00±0.26	2.33±0.21	2.00±0.26	1.83±0.31	1.33±0.21	14.82±0.87*
Metoclopramide (1.0 mg/kg) + scopolamine (0.56 mg/kg)	4.17±0.40	3.17±0.31	3.00±0.26	3.50±0.71	3.33±0.61	2.33±0.21	19.50±0.95**,$
					One-way ANOVA	F	15.16
						df	5,30
						P	<0.001

*P < 0.01; **P< 0.001 versus saline group. The values are mean±SEM. (n=6 in each group). [P values were calculated for total working memory errors versus saline group]. $P<0.05, compared to scopolamine (0.56 mg/kg)[$F_{2,15}$=5.63, p=0.015]. @All drugs were given intraperitoneally.

0.56 mg/kg, in comparison to the saline group. (P<0.0001) (Table 1).

The effect of metoclopramide was investigated on the scopolamine-induced working memory errors. Co-administration of metoclopramide (1.0 mg/kg) with scopolamine (0.56 mg/kg) resulted in a significant increase in the working memory errors, in comparison to the scopolamine alone group (P<0.05) (Table 1).

The rats co-administered with metoclopramide (5.0 mg/kg) and scopolamine (0.56 mg/kg) failed to run on the three-panel runway apparatus within the cut off time period of 2 min and were not included in the table.

Latency period: Intraperitoneal scopolamine (0.1- 0.56 mg/kg) resulted in an increase in the latency period in comparison to the saline group (Table 2). Scopolamine in the dose of 0.32 mg/kg (P<0.05) and 0.56 mg/kg (P<0.01) resulted in a significant increase in the total latency period of the session in comparison to the saline group (Table 2).

The effect of metoclopramide was investigated on the scopolamine-induced increase in the latency period. Co-administration of metoclopramide in the doses of 0.1, 1.0 mg/kg with scopolamine (0.56 mg/kg) did not increase the latency period in comparison to the scopolamine treated group (Table 2).

DISCUSSION

Working memory allows animals to remember information that is useful for a single session of an experiment but not for subsequent sessions[2]. Three-panel runway apparatus, a modification of Hill's apparatus, is designed to study working memory in rats where more than 2 choices were available for the rat at each choice point and thus is considered to be superior in assessing the discriminatory paradigm[6].

Table 2. Effect of scopolamine and metoclopramide on latency period in albino rats on three-panel runway apparatus.

Group	Latency periods in each trial (sec)						Total
	1	2	3	4	5	6	
Saline (control)	18.39±2.28	9.94±2.51	6.57±1.42	10.65±3.52	7.20±2.17	4.70±0.99	57.45±7.38
Scopolamine (0.1 mg/kg)	23.48±1.78	13.85±1.97	9.52±1.61	6.57±0.18	6.43±0.55	5.72±0.19	65.57± 3.60
Scopolamine (0.32 mg/kg)	43.68±8.21	24.24±4.04	15.80±2.08	11.34±1.50	9.12±1.13	8.27±0.86	112.45±14.18*
Scopolamine (0.56 mg/kg)	54.99±3.63	28.58±5.98	20.15±6.12	21.64±2.72	16.23±4.00	10.38±1.52	151.97±10.68**
Metoclopramide (0.1 mg/kg) + scopolamine (0.56 mg/kg)	53.73±3.71	27.15±4.20	23.11±4.44	20.46±3.44	15.89±3.65	12.19±0.87	152.53±13.13**
Metoclopramide (1.0 mg/kg) + scopolamine (0.56mg/kg)	82.63±6.59	25.44±5.81	23.56±5.84	16.78±3.23	11.12±2.39	7.62±1.20	167.15±15.12**
					One-way ANOVA	F	13.16
						df	5, 30
						P	<0.01

*P <0.05; **P <0.01 versus saline group. The values are mean±SEM. (n = 6 in each group). [P values were calculated for total latency period versus saline group].

In the present study, all the trained rats had acquired a steady state (basal average score of <12 errors per session) after repeated acquisition procedure[6]. In the present study on the three-panel runway apparatus, rats could be trained to reach a steady state in about 15 sessions. The prime determinant of working memory deficits in three-panel runway apparatus is the number of working memory errors committed by the rodent in a session. Latency period however, is a weak indicator of working memory in this apparatus, as latency period would in turn depend on, the degree of appetite, speed of the rat and more importantly, the number of training sessions before the test run.

In the present study scopolamine treatment in rats on the three-panel runway apparatus resulted in no significant increase in the number of errors in the first trial, when compared to the saline treated group (Table 1). This is in accordance with other studies, where amnesic doses of scopolamine caused no significant increase in the first trial error responses in comparison to the saline group[6,7].

In our study scopolamine administration in the dose of 0.1 mg/kg did not result in any significant increase in the number of errors or latency periods in a session on the three-panel runway apparatus when compared to the saline control. This finding, which was similar to the study by Ohno *et al* on the three panel runway apparatus[7].

In our study scopolamine administration in the dose of 0.32 mg/kg did not result in any significant increase in the number of errors, a finding similar to the study reported by Furuya *et al* [6]. However, the same dose resulted in a significant increase in the latency period in our study. Ohno *et al* [7] reported a significant increase in both the number of errors and latency period with the dose of 0.32 mg/kg of scopolamine.

In the present study scopolamine administration in the dose 0.56 mg/kg resulted in significant increase in both working memory errors and latency period. Scopolamine in the dose of 0.56 mg/kg was conclusively shown to cause working memory deficits on the three-panel runway apparatus[6].

Galeotti *et al* [13] had reported that metoclopramide reversed, dicyclomine-induced impairment of passive avoidance task, possibly explained by 5-HT_3 antagonist and 5-HT_4 agonist properties of metoclopramide. However, in our study metoclopramide failed to reverse, scopolamine induced working memory impairment. On the contrary, the number of errors were increased significantly by metoclopramide. Possible explanation could be that the three-panel runway task is an appetite motivated reward-learning model. As dopamine neurotransmission plays an important role in incentive motivational (reward) learning[3], metoclopramide, [dopamine (D2) receptor antagonist] could have inhibited the incentive learning, resulting in working memory deficits on the three-panel runway apparatus. Furthermore, a D2 receptor antagonist raclopride was reported to cause working memory deficits in the appetite motivated radial arm maze[15] and impaired performance in aversively motivated T-maze[16]. Microdialysis studies revealed that D2 receptor antagonist inhibit ACh release in the ventral hippocampus[16].

To summarize, the present study revealed that scopolamine, a muscarinic receptor antagonist, induced working memory impairment on the three-panel runway apparatus and metoclopramide treatment significantly aggravated the working memory impairment produced by scopolamine.

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Review

Scopolamine model of dementia: electroencephalogram findings and cognitive performance

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Abstract

Background Memory and cognitive functions are known to decline with advancing age. Studies have suggested that this may be due to a decrease in cholinergic function in the brains of elderly people. This review aims to assess studies documented in the literature dealing with the 'scopolamine model' of dementia.

Methods Sources included MedLine searches from the last 10 years (search for 'scopolamine model', 'dementia', 'electroencephalogram', 'cognition') and references from original and review articles. The aim was to include human and animal studies occupying the cholinergic hypothesis in cognitive dysfunction. Electroencephalographic (EEG) and cognition findings were considered.

Results Scopolamine influences delta, theta, alpha and beta activity in EEG and partially mimics the EEG changes found in patients with senile dementia or dementia of the Alzheimer type. Effects on different cognitive functions have been extensively documented.

Conclusion Scopolamine produces similar memory deficits seen in the elderly, but the drug cannot induce the full range of deficits seen in patients with Alzheimer's disease. Various aspects of memory were unaffected by scopolamine administration. Memory improvements in elderly subjects can be achieved after cholinergic stimulation.

Keywords Cholinergic hypothesis, cognition, electroencephalogram, scopolamine model.
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Introduction

Age-related changes in the central nervous system (CNS) have been observed in many neurotransmitter systems. This review is focused on changes in the central cholinergic system.

Cholinergic neurons in the CNS are believed to be involved in learning and memory [1–3]. Studies indicate that many otherwise healthy persons show a decline in cognition in later life. Animal and human studies have suggested that one major factor in age-related senile CNS dysfunction and the early states of Alzheimer's disease may be a disruption in the cholinergic neurotransmitter system [4–8]. One model, based upon this 'cholinergic hypothesis', is provided by the use of the muscarinic receptor antagonist scopolamine [9]. Scopolamine is an anticholinergic agent approved for use in the prevention of nausea and vomiting associated with motion sickness. Owing to its anticholinergic activity, scopolamine induced a pattern of memory and cognitive deficits in young volunteers that was markedly similar to the performance of elderly subjects [7,10,11]. An increase in delta power [12,13] and a decrease in alpha and beta frequencies [14] in the electroencephalogram (EEG) have been described after administration of scopolamine in healthy volunteers. Studies in rats have shown that administration of muscarinic antagonists and lesions of the nucleus basalis of Meynert, the major origin of the cortically projecting cholinergic fibres, is associated with an increase in power of slow waves in the EEG [2,15]. Thus, scopolamine has suggested to be a psychopharmacological model drug of CNS ageing.

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Central cholinergic function and age-related changes

Muscarinic receptors have been identified in many parts of

the brain [16,17]. The majority of cholinergic neurons in the CNS can be subdivided into four major groups: (a) cholinergic basal nuclear complex, (b) cholinergic interneurons, (c) mesopontine tegmental cholinergic group and (d) somatic motoneurons [18]. Opposing reports exist concerning changes in the concentration of muscarinic acetylcholine receptors in patients with Alzheimer's disease. Receptor concentration may be changed [19] and has found to be decreased [20] or unaltered [21] in autopsy samples. Controversy also exists regarding the question of which transmitter system suffers the most with normal ageing and whether this pattern differs in the brains of patients with Alzheimer's disease [2]. Studies in this patient population have shown a severe loss of neurons in the nucleus basalis of Meynert [9]. These findings suggest that the decrease in cortical choline acetyltransferase found in Alzheimer's patients may reflect a specific loss of cholinergic input to the cortex [2] because the nucleus basalis of Meynert is believed to provide the primary cholinergic input to the cortical mantle [22–24]. The enzyme choline acetyltransferase is involved in the synthesis of acetylcholine and is a specific marker for cholinergic neurons. Bartus *et al.* [2] thoroughly reviewed human and animal studies that investigated choline acetyltransferase activity in the brain. Most animal data, in agreement with the findings in human literature, demonstrated a small decrease in choline acetyltransferase activity with age. Investigations in aged rodents have described an age-related decrease in the density of muscarinic receptors with no change in affinity [25–29].

The results suggest that normal ageing is associated with decreased muscarinic receptor density in the brain. Age-related changes in the central cholinergic system are reflected in a decreased functional activity of cholinoreceptive neurons. If changes in the cholinergic system are involved in memory deficiency observed in the elderly, pharmacological disruption of the cholinergic system should induce similar changes in young subjects.

In addition to the acetylcholine system, other neurotransmitters are known to be involved in learning and memory. Experimental data suggest that stimulation of serotonergic neurotransmission impairs behavioural performance, whereas inhibition of the system enhances performance [30]. Dunnett & Robbins [31] suggested that the dopaminergic system is related to visual processing and attention. Excitatory amino acid receptor agonists such as glutamate showed adverse effects: long-term potentiation has been suggested to be the physiological correlate of memory formation [32]. On the other hand, high levels of glutamate are neurotoxic [33]. A glutamatergic deficit has been found in patients with Alzheimer's disease [34,35]. The interactive role between these neurotransmitters is poorly understood [36]. It has been suggested that Alzheimer's dementia results from complex neuron alterations rather than simply reflecting acetylcholine impoverishment [37].

Further descriptions will be focused on the cholinergic hypothesis.

Scopolamine model of dementia

The cholinergic system is involved in the storage and retrieval of information during new learning [6]. The blockade of central muscarinic receptors induces a memory deficit in young subjects that is similar to that occurring in elderly subjects [2]. Scopolamine, an anticholinergic agent, reduces the effective action of a given concentration of acetylcholine at the synapse without actually changing the concentration itself. Scopolamine occupies some of the receptor sites on the post-synaptic membrane without producing depolarization [6], thus reducing the effectiveness of acetylcholine. The characterization of the interaction of scopolamine with the muscarinic acetylcholine receptor in the CNS indicated a two-step process with an initial binding of ligand to receptor followed by isomerization of the receptor–ligand complex to a higher affinity form [38].

It was postulated that scopolamine also interferes with other neurotransmitter systems [39] and affects the regional cerebral blood flow measured during the performance of memory tasks [40].

In human studies the model substance scopolamine has been used in doses between 0·25 and 1·0 mg given by parenteral administration [11]. Like atropine, there is a large variability in maximum plasma concentration (C_{max}) and the time to reach C_{max} (t_{max}) after intramuscular administration [41]. Because the elimination of scopolamine, like that of atropine (elimination half-life 2·4 ± 1·4 h [41]), is quite rapid, single doses of scopolamine have only short-lasting effects. Central pharmacodynamic effects peak between 1 and 3 h and disappear after 5–6 h [42].

Scopolamine-induced changes in the EEG

Drug-induced EEG changes are generally accepted as a method of predicting the clinical potency of drugs. It has been shown that the human EEG can serve as a sensitive and valid measurement system in this regard [43]. Investigations in young healthy volunteers who received scopolamine have been shown that the drug influences the delta, theta, alpha and beta frequency bands in the EEG. Various studies have shown an increase in the delta and theta frequency bands [13,42] and a decrease in the alpha [13,14,36,44] and beta frequency bands [45]. Sannita *et al.* [13] have shown a dose-related increase in relative power in the 0·5–3·5 and 4·0–7·5 Hz spectral segments (delta and theta frequency bands) and a concomitant reduction in the relative power in the 8·0–13·5 Hz frequency band (part of the alpha and beta frequency bands). We also observed an increase in spectral power density in the delta frequency band (1·25–4·50 Hz) 1 h after subcutaneous administration of 0·6 mg of scopolamine compared with baseline [42]. In a recent study in young healthy male volunteers, an increase in spectral delta (1·25–4·50 Hz) and theta power density (4·75–6·75 Hz) and a decrease in spectral $alpha_1$ (7·00–9·50 Hz), $alpha_2$ (9·75–12·50 Hz), $beta_1$ (12·75–18·50 Hz) and $beta_2$ power

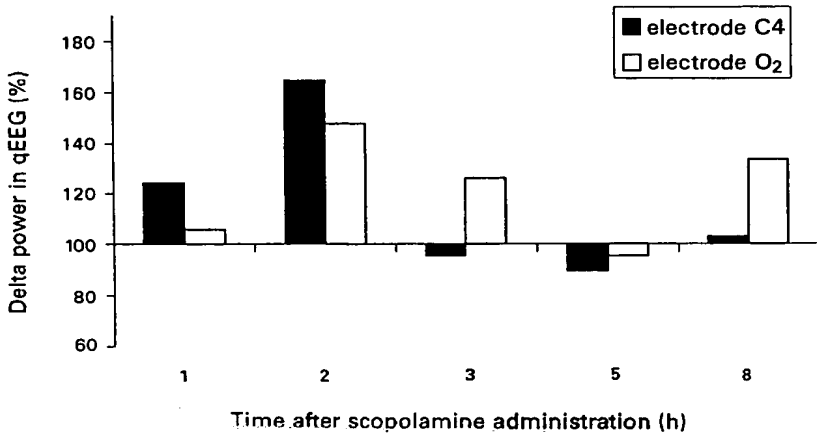

Figure 1 Changes in delta power in quantitative electroencephalogram after subcutaneous injection of scopolamine 0·6 mg in 10 healthy male subjects. Data represent changes over placebo (%) in central (C_4) and occipital (O_2) electrode position. Values are medians.

density (18·75–35·00 Hz) were found in qEEG after subcutaneous administration of scopolamine compared with placebo [45]. The changes in delta and alpha$_2$-power were statistically significant (Figs 1 and 2), whereas changes in theta, alpha$_1$, beta$_1$ and beta$_2$- power did not reach statistical significance and could only be explained as a trend. Scopolamine also influenced the alpha frequency band: studies have shown a decrease in the 8·0–13·9 Hz power after subcutaneous injection of 0·6 mg of scopolamine [14] and a significant reduction in the 8·5–12·0 Hz power after intramuscular administration of 0·5 and 0·75 mg of scopolamine [44]. Meador *et al.* [36] also described a reduction in alpha power induced by scopolamine. In a recent study, we observed a decrease in beta power in addition to a decrease in alpha power, but the changes in the beta frequency band did not reach statistical significance (unpublished data).

Studies in patients with Alzheimer's disease and age-matched control subjects treated with scopolamine have also shown an increase in delta activity and a decrease in alpha amplitude [12]. Several investigators have described an increase in delta and theta power and a decrease in alpha power in patients with senile dementia or Alzheimer's disease compared with age-matched control subjects [46,47]. Horie *et al.* [48] distinguished different changes in EEG and different stages of Alzheimer's disease. Studies in rats have shown that administration of muscarinic antagonists and lesions of the nucleus basalis of Meynert, the major origin of the cortically projecting cholinergic fibres, were associated with an increase in EEG delta power [2,15]. These findings support the inverse relationship between cholinergic involvement and delta activity in rats. A similar relationship probably exists in humans.

Scopolamine also influences scalp-recorded visual evoked potentials (VEPs). VEPs as well as the background EEG are the products of processes intrinsic to the cortex and of modulation from subcortical structures [44]. It has been suggested that VEP is a relevant issue in understanding neural mechanisms and neurotransmission in the visual system [44]. Scopolamine produces an increase in the latency of the flash-VEP P_2 component in young normal subjects but does not affect the pattern reversal VEP [49]. These results are similar to those found in Alzheimer's disease. Sannita *et al.* [44] also described a reduction in flash-VEP P2-N3 amplitude after intramuscular administration of scopolamine (doses ranged from 0·25 mg to

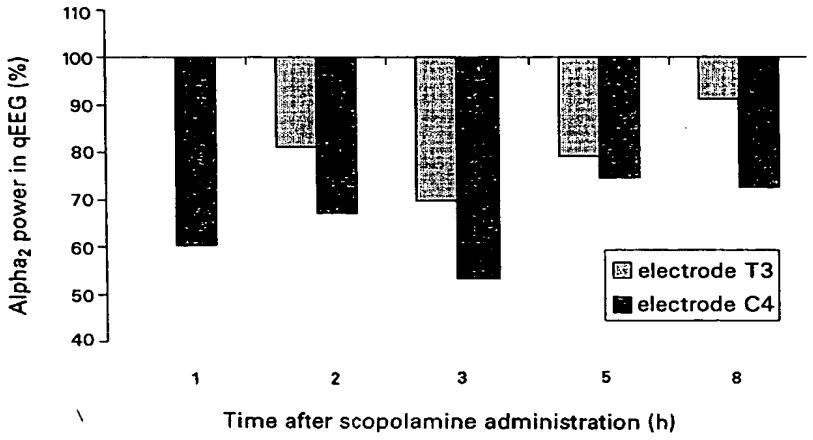

Figure 2 Changes in alpha$_2$ power in quantitative electroencephalogram after subcutaneous injection of scopolamine 0·6 mg in 10 healthy male subjects. Data represent changes over placebo (%) in central (C_4) and parietal (T_3) electrode position. Values are medians.

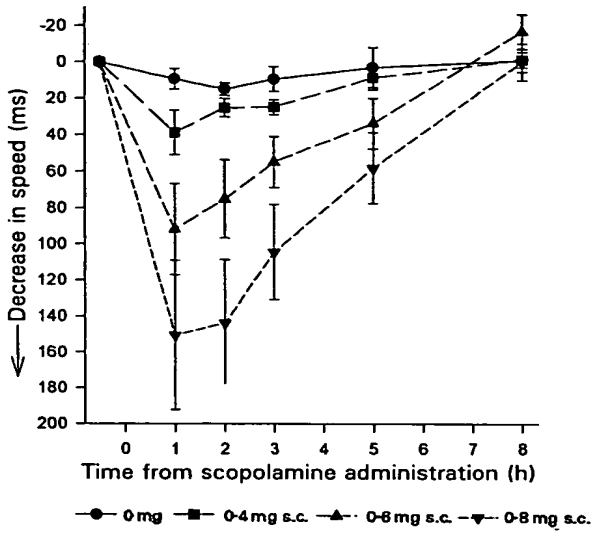

Figure 3 Changes in simple reaction time (decrease in speed in ms) after subcutaneous administration of three different doses of scopolamine.

0·75 mg). Brodie *et al.* [50] suggest that a decline in visual processing capacities in patients with Alzheimer's disease is in line with the continuous degeneration of neuronal populations involved with vision.

Scopolamine-induced changes in cognitive performance

The effects of scopolamine on different cognitive functions in young healthy subjects have been extensively documented. Wesnes [11] described a pronounced effect of scopolamine after subcutaneous administration on the efficiency of detection and processing information in tests of visual vigilance, choice reaction, letter cancellation and logical reasoning. Several investigators [8,14,51–55] were able to obtain similar results on cognitive functions studied with different methods. It has been suggested that acetylcholine seems to be more involved in attentional processes than in learning and memory [56]. Wesnes & Warburton [52] concluded from a study in healthy volunteers that the central cholinergic system plays an important role in the performance of tasks with minimal memory requirement. This emphasizes the findings of a study that we recently performed in healthy volunteers: scopolamine significantly affected simple reaction time (Fig. 3), number matching, choice reaction time and memory scanning tasks, but no differences between scopolamine and placebo were found in word recognition and delayed word recall [45]. The administration of scopolamine also induced sedative effects, accommodation disturbances and dryness of the mouth. These are well-known classical anticholinergic adverse reactions.

Animal tests of cognitive function have demonstrated similar results [57–61]. On the other hand, Godding *et al.* [62] could not demonstrate an effect of scopolamine on working memory in rats. Flood & Cherkin [39] speculate that counteraction of scopolamine-induced amnesia is not specific to the cholinergic system. Stone *et al.* [58] have shown that adrenaline and glucose can significantly reduce scopolamine-induced amnesia in mice. It has been suggested that serotonin and acetylcholine interact to allow normal cognitive function in the brain [60].

Scopolamine leads to memory impairment in young subjects, mimicking some of the changes observed in elderly subjects. On the other hand, scopolamine cannot induce the full range of deficits seen in patients with Alzheimer's disease [63]. Various aspects of memory are unaffected by scopolamine administration. The size of the memory deficits after scopolamine is much smaller than that seen in Alzheimer's disease [64]. The different changes in attention, learning and memory may reflect the hypothesis that scopolamine interacts with different neurotransmitter systems. Scopolamine may affect the different systems equally, but the consequences of affecting one system may overshadow the effects on another. This could also reflect the different findings of distinct doses of scopolamine.

Conclusion

Extensive research has established that the central cholinergic system is involved in the storage and retrieval of information during new learning. Based upon this 'cholinergic hypothesis', one model is provided by the use of scopolamine. Scopolamine is a non-selective post-synaptic muscarinic receptor antagonist that blocks the stimulation of post-synaptic receptors and causes memory deficits when administered to young healthy subjects. Scopolamine can also induce an increase in delta and theta frequency bands and a decrease in alpha frequency bands in the EEG in young healthy volunteers. An increase in delta and theta power and a decrease in alpha power reflects the changes seen in patients with senile dementia or Alzheimer's disease. Studies in rats have shown that administration of muscarinic antagonists is associated with an increase in power of slow waves in EEG. Scopolamine-induced memory and other cognitive deficits in young subjects mimic some of the changes occurring in elderly subjects. On the other hand, it should be considered that cognition is not a single homogenous function that might be modelled in its entirety by a single procedure [57]. It should be also noted that the loss of cholinergic input into the cortex observed in patients with Alzheimer's disease [2] could lead to compensatory changes in the brain. Therefore, we speculate that chronic administration of scopolamine could better mimic these changes. Owing to ethical aspects in human studies, this hypothesis can be verified only in animal investigations.

Scopolamine also affects other central neurotransmitter systems, decreases the regional cerebral blood flow and

causes a depression in regional central glucose metabolic rates [65]. This may support the hypothesis that scopolamine may affect different systems, but the consequences of affecting one system may overshadow the effects on others.

In summary, the scopolamine model of dementia reflects only parts of the changes found in age-related senile CNS dysfunctions. The central cholinergic system seems to be more involved in attentional processes than in learning and memory processes. It should be considered that age-related changes in the CNS have been observed in many neurotransmitter systems and that scopolamine affects different central neurotransmitter systems, central blood flow and glucose metabolic rate. It seems that more specific cholinergic damage using a highly specific cholinergic toxin could be a better tool to characterize the special role of central cholinergic system in learning and memory.

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Improving Effects of Huperzine A on Spatial Working Memory in Aged Monkeys and Young Adult Monkeys with Experimental Cognitive Impairment

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ABSTRACT

Our previous studies demonstrated that huperzine A, a reversible and selective acetylcholinesterase inhibitor, exerts beneficial effects on memory deficits in various rodent models of amnesia. To extend the antiamnesic action of huperzine A to nonhuman primates, huperzine A was evaluated for its ability to reverse the deficits in spatial memory produced by scopolamine in young adult monkeys or those that are naturally occurring in aged monkeys using a delayed-response task. Scopolamine, a muscarinic receptor antagonist, dose dependently impaired performance with the highest dose (0.03 mg/kg, i.m.) producing a significant reduction in choice accuracy in young adult monkeys. The delayed performance changed from an average of 26.8/30 trials correct on saline control to an average of 20.2/30 trials correct after scopolamine administration. Huperzine A (0.01–0.1 mg/kg, i.m.) significantly reversed deficits induced by scopolamine in young adult monkeys on a delayed-response task; performance after an optimal dose (0.1 mg/kg) averaged 25.0/30 correct. In four aged monkeys, huperzine A (0.001–0.01 mg/kg, i.m.) significantly increased choice accuracy from 20.5/30 on saline control to 25.2/30 at the optimal dose (0.001 mg/kg for two monkeys and 0.01 mg/kg for the other two monkeys). The beneficial effects of huperzine A on delayed-response performance were long lasting; monkeys remained improved for about 24 h after a single injection of huperzine A. This study extended the findings that huperzine A improves the mnemonic performance requiring working memory in monkeys, and suggests that huperzine A may be a promising agent for clinical therapy of cognitive impairments in patients with Alzheimer's disease.

Alzheimer's disease (AD) is a slowly progressive neuropsychiatric illness, principally characterized by memory deficits. There is a substantial body of experimental work in animals and humans suggesting that the cholinergic mechanism plays an essential role in AD (Davies and Maloney, 1976; Bartus et al., 1982; Coyle et al., 1983). Dysfunction in cholinergic mechanisms may contribute to age-related memory impairments. The retrograde loss of the cholinergic system from the basal forebrain is the most common and the most severe neurochemical consequence of the disease (Susan, 1997). The cholinergic neuron clusters of the basal forebrain innervate the hippocampus and areas of association in the cortex involved in higher processes such as long-term memory, working memory, and attention. In these structures, the concentration of choline acetyltransferase (ChAT) decreased, accompanied by the impaired ability of high-affinity choline transport and synthesis of acetylcholine (ACh). The severity of memory impairments seen in AD is consistent with dysfunction of the cholinergic system (Coyle et al., 1983). Many attempts have been made to correct the cholinergic deficiency at various levels of cholinergic functioning to reduce, if not cure, some of the major cognitive disturbances of AD patients. Some cholinomimetic agents have been shown to improve age-related cognitive impairments. Among various cholinomimetic drugs, the acetylcholinesterase (AChE) inhibitor as a palliative agent in the treatment of AD has been the most promising so far (Parnetti et al., 1997). Physostigmine and tacrine have shown some clinical efficacy in AD patients (Mohs et al., 1985; Summers et al., 1986). They are not, however, ideal drugs for clinical use due to the short duration of action, the low bioavailability, and the frequent side effects with physostigmine (Winblad et al., 1991) and dose-dependent hepatotoxicity of tacrine (Watkins et al., 1994). Thus the search for a new cholinesterase inhibitor (ChEI) with properties that could overcome the limitations in the use of physostigmine and tacrine is still ongoing (Giacobini, 1997).

In addition to the central cholinergic system, other transmitter systems such as the monoaminergic system are

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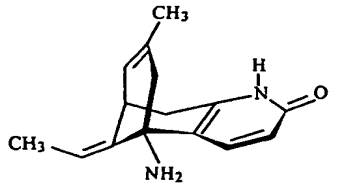
ABBREVIATIONS: AD, Alzheimer's disease; ChEI, cholinesterase inhibitor; ChAT, choline acetyltransferase; NE, norepinephrine; DA, dopamine; PFC, prefrontal cortex; 1-ANOVA-R, one-way analysis of variance with repeated measures.

thought to participate in causing dementia in AD patients (Palmer and DeKosky, 1993). There is evidence of interaction between cholinergic and monoaminergic systems in the control of cognitive cortical function (Riekkinen et al., 1990). The positive clinical effect of ChEIs such as tacrine has been related to stimulation of both cholinergic and monoaminergic systems (Alhainen et al., 1993). Therefore, the nootropic effects of ChEIs may involve cholinergic mechanisms as well as monoaminergic mechanisms.

Huperzine A, a *Lycopodium* alkaloid isolated from the Chinese herb *Huperzia serrata* (Thunb) Trev, is a reversible and selective AChE inhibitor. The experiments showed that huperzine A can produce a long-term inhibition of AChE activity in rat brains and a sustained increase of ACh levels in the central nervous system (Tang et al., 1989). Compared with physostigmine, tacrine, and galanthamine, the AChE inhibitory effect of huperzine A is more potent, its selectivity for AChE other than butyrylcholinesterase is better, and its duration of inhibition is longer; its bioavailability is higher but the side effects are less (for review see Tang, 1996). It has been reported that huperzine A can produce a dose-dependent increase of other transmitters such as norepinephrine (NE) and dopamine (DA) in the rat cortex with either systemic or local intracerebral administration (Zhu and Giacobini, 1995). The previous studies in rodents showed that huperzine A improves performance in a variety of paradigms including spatial memory tasks (Tang, 1996) such as Y-maze (Tang et al., 1986; Lu et al., 1988) and the radial-arm maze (Xiong and Tang, 1995; Cheng et al., 1996). The duration of improving effects of huperzine A on learning and memory retention processes was longer than that of physostigmine or tacrine (Tang et al., 1994).

Scopolamine, a muscarinic receptor antagonist, has been shown in numerous studies to impair learning and memory under a variety of testing conditions, not only in small animals (Spencer and Lal, 1973), but also in monkeys (Ogura and Aigner, 1993; Rupniak et al., 1989) and in human (Ghoneim and Mewaldt, 1977; Rusted and Warburton, 1988); some of these impairments reflect neuropsychological similarities with the demented states in patients with AD (Molchan et al., 1992). The aged monkey is also a good candidate for studies of AD, because its behavioral impairments are similar to those that are characteristic of elderly human (Bartus, 1979). In particular, the similarities of neurochemical changes in aged monkeys with those in humans indicate that the aged monkeys may be a useful model for investigation of the age-associated transmitter abnormalities which are similar to those that occur in human (Wenk et al., 1989).

The aim of this study was to extend the findings as to whether huperzine A can improve the memory impairments in aged monkeys with a naturally occurring ACh decrease and in young monkeys with an experimental disruption of cholinergic system using scopolamine. The chemical structure of huperzine A is shown in Fig. 1.



(-)-Huperzine A

Fig. 1. Chemical structure of huperzine A.

Materials and Methods

Subjects. The subjects in this study consisted of eight rhesus monkeys (*Macaca mulatta*). Four young female monkeys (three were approximately 6–7 years old; one was approximately 4 years old) were used to evaluate the effect of huperzine A on scopolamine-induced memory impairments. The four aged monkeys (two females and two males approximately 16–18 years old) were used to study the effect of huperzine A on age-related memory deficits. Because actual birth dates were unavailable, ages were estimated on the basis of prior breeding and behavioral testing records, dental records, and general appearance. Rhesus monkeys in captivity have been reported to live 20 to 25 years and longer. All young adult subjects were drug-naive, whereas all aged subjects had prior behavioral testing experience, but none had been involved in drug tests in the 1 year preceding the present investigation. All animals were housed individually under standard laboratory conditions. Feeding occurred immediately after cognitive testing. Daily supplements of fruits and vitamins were also given. Water was available ad libitum.

Delayed-Response Testing. Monkeys were tested in the Wisconsin General Testing Apparatus. The test tray contained a left and a right food well spaced 15 cm apart. An opaque screen was lowered to separate the monkey from the test tray. For testing sessions, the test panel was attached to the home cage. While testing was in progress, the light in panel was on so that the monkey could see clearly what happened in the panel. Highly palatable food rewards (e.g., peanuts, raisins, or sugar chips) were used during testing to minimize the need for dietary regulation. The monkeys were tested daily at the same time of day in a quiet room by a trained observer. Using these conditions, no problems with motivation were evident.

The monkeys had previously been trained on the two-well, delayed-response task. During delayed response, the animal watched as the experimenter baited one of two food wells. The food wells were then covered with identical cardboard plaques, and an opaque screen was lowered between the animal and the test tray for a specified delay. At the end of this delay, the screen was raised and the animal was allowed to choose. Reward was quasi-randomly distributed between the left and right wells over the 30 trials that made up a daily test session. During the initial training phase, delays were held constant during a daily session and were gradually increased from 0 s according to a step-wise procedure over the 1000 trials.

Following the 1000 trials, the monkeys were prepared for drug testing. To observe the effects of drug on memory capacity, the animals were trained on a variable delayed-response task in which five different delay lengths were distributed over the 30 trials that made up the daily test session. For four aged monkeys, delays were adjusted until the animals exhibited stable baseline performance of approximately 67% correct. For example, the range of delays for aged monkey no. 35 was 0, 6, 12, 18, and 24 s. All aged subjects performed perfectly at 0-s delays and exhibited increasing difficulty with progressively longer delays, a pattern consistent with memory impairment. In young adult monkeys, delays were chosen to produce performance levels of about 90% correct out of 30 trials. For example, the range of delays was 0 to 20 s for monkey no 19 and 0 to 8 s for monkey no. 36. The 0-s delay consisted of lowering the screen and immediately raising it again. Once performance was demonstrated to be stable at this baseline, drug treatment was initiated.

Drug Administration. Huperzine A (provided by the Department of Phytochemistry, Shanghai Institute of Materia Medica, Chinese Academy of Sciences) and scopolamine hydrobromide (Sigma Chemical Co., St. Louis, MO) were both dissolved in sterile 0.9% saline before injection. Scopolamine or saline and huperzine A or

saline were injected i.m. 30 min and 20 min, respectively, before delayed-response testing. The injection volume was kept constant at 0.1 ml/kg irrespective of dose.

The doses of scopolamine were 0.01, 0.02, and 0.03 mg/kg; huperzine A was coadministered with the highest dose of scopolamine (0.03 mg/kg) to young adult monkeys. At this dose of scopolamine, all the young subjects exhibited significant memory impairments so that there was enough room to test the effects of huperzine A. The doses of huperzine A were 0.001, 0.01, 0.1, and 0.2 mg/kg for young subjects and 0.0001, 0.001, 0.01, and 0.1 mg/kg for aged subjects. A wide range of doses was selected to ensure the optimal dose within it for each monkey.

Drugs were administered no more than twice per week (Monday-Saturday), and at least 3 days separated test sessions. Control injections of saline alone were given the day before each drug testing to assure that the performance was back to the baseline level. The experimenter testing the monkeys was unaware of the drug treatment conditions.

Data Statistics. Delayed-response performance on drug was compared with matched placebo (saline) control performance for the same week. Because the animals served as their own controls, statistical analyses employed repeated measures designs: one-way analysis of variance with repeated measures (1-ANOVA-R), and, if appropriate, followed by post hoc tests. The level of significance was $P < .05$.

Results

Effects of Scopolamine on Delayed-Response Performance in Young Adult Monkeys. Scopolamine at the doses of 0.01, 0.02, and 0.03 mg/kg produced a dose-related impairment in the performance of young monkeys [1-ANOVA-R: $F(3,9) = 6.66$, $P = .0116$] with the highest dose of scopolamine causing a significant disruption of choice accuracy in all of the young animals [$F(1,3) = 43.78$, $P = .0070$] (Fig. 2). After the 0.03-mg/kg dose, performance at all retention intervals was impaired but the magnitude of this effect increased as the retention interval lengthened (Fig. 3) [9.7 ± 4.7%, 10.3 ± 7.2%, 25.7 ± 8.7%, 30.5 ± 14%, and 33.3 ± 2.5% decreases at A (0 sec), B, C, D, and E delays respectively]. 1-ANOVA-R suggested no significant effect of scopolamine at 0-s delays [$F(1,3) = 4.42$, $P = .1263$], but significant effect was found at the longest delays [$F(1,3) = 182.65$, $P = .0009$]. At the highest dose of scopolamine (0.03 mg/kg), some signs of the side effects of cholinergic antagonism, such as a slower rate of chewing than usual and pupillary dilation, were observed.

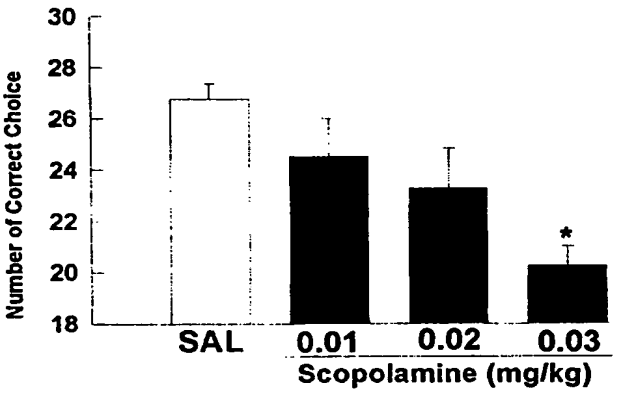


Fig. 2. Effects of scopolamine on delayed response task in young adult monkeys ($n = 4$). Saline or scopolamine administered i.m. 30 min before testing. Values represent mean ± S.E.M. number of trials correct out of a possible 30 trials. *$p < .05$ versus saline control.

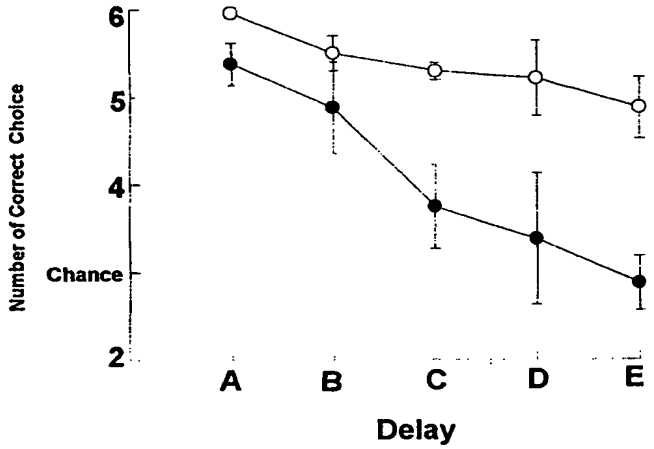


Fig. 3. Effects of scopolamine (0.03 mg/kg, ●) compared with saline (○) on delayed response performance for each of five delay intervals (A, B, C, D, and E) used in each testing session in young adult monkeys ($n = 4$). The A delay was always 0 s; the B, C, D, and E delays were incrementally increasing delays (e.g., 5, 10, 15, and 20 s) individually selected for each animal to produce an overall baseline performance of about 67% correct (see *Materials and Methods*). Values represent mean ± S.E.M. number correct out of a possible six trials at each delay interval.

Effects of Huperzine A on Scopolamine-Induced Deficit of Delayed Performance in Young Adult Monkeys. Huperzine A markedly improved the delayed-response performance of scopolamine-treated monkeys (Fig. 4A) [1-ANOVA-R: $F(4,12) = 14.3$, $P = .0002$]. The dose-response curve was bell-shaped with the maximum improvements at 0.1 mg/kg [15 ± 2.9% increase, 1-ANOVA-R: $F(1,3) = 27.0$, $P = .0138$, compared with scopolamine control]. Neither the lowest nor the highest doses had effects [$F(1,3) = 2.45$, $P = .23$ for 0.001 mg/kg; $F(1,3) = 2.46$, $P = .21$ for 0.2 mg/kg] (e.g., young monkey no. 36, Fig. 4B). The beneficial effects of huperzine A were most evident at the longest delays [27.1 ± 2.5% increases, $F(1,3) = 10.50$, $P = .048$].

Effects of Huperzine A on Delayed-Response Performance in Aged Monkeys. Administration of huperzine A to aged monkeys produced a significant effect on delayed-response performance [1-ANOVA-R: $F(4,12) = 11.26$, $P = .0005$]. As can be seen in Fig. 5A, huperzine A produced a bell-shaped dose-response curve similar to the one described above in scopolamine-treated young monkeys. There were variances between the performance of four aged subjects. Of these four doses (0.0001−0.1 mg/kg), the best dose was 0.001 mg/kg for two monkeys, and for the remaining two animals the best dose was 0.01 mg/kg (e.g., monkey no. 34, Fig. 5B). The improvements following the best doses were most apparent at two longer delays (Fig. 6) [$F(1,3) = 16.94$, $P = .026$; $F(1,3) = 12.63$, $P = .0380$, respectively]. However, performance at 0-s delays did not change between monkeys on saline and on huperzine A [$F(1,3) = .36$, $P = .59$]. These results are consistent with changes in cognitive performance rather than a nonspecific performance variable, which would be expected to disrupt performance after the 0-s delay control trials.

During the sessions conducted 24 h after huperzine A injection at the dose of 0.01 mg/kg and 0.1 mg/kg, the improving performance remained evident (Fig. 7). Moreover,

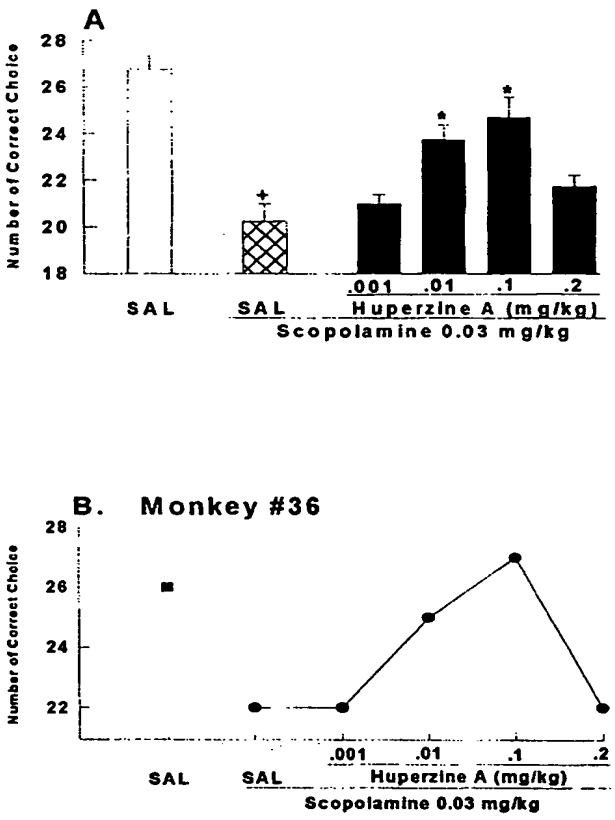


Fig. 4. Effects of huperzine A on the deficit of delayed-response performance induced by scopolamine in young adult monkeys. Saline or huperzine A was administered i.m. 20 min before testing. Scopolamine was administered 30 min before testing. A, huperzine A produced a dose-related improvement in the delayed-response performance of young monkeys (n = 4). Values represent mean ± S.E.M. number of trials correct out of a possible 30 trials. $^{+}p < .05$ versus saline control; $^{*}p < .05$ versus scopolamine control. B, effects of monkey no. 36. Values represent mean number of trials correct out of a possible 30 trials.

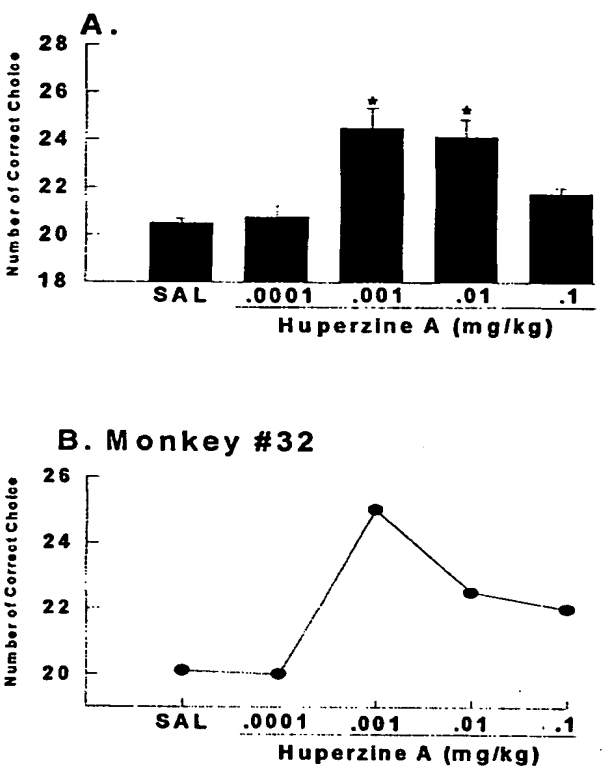


Fig. 5. Effects of huperzine A in aged monkeys. Saline or huperzine A was administered i.m. 20 min before testing. A, huperzine A produced a dose-related improvement in the delayed-response performance of aged monkeys (n = 4). Values represent mean ± S.E.M. number of trials correct out of a possible 30 trials. B, effects of huperzine A in monkey no. 32. Values represent mean number of trials correct out of a possible 30 trials.

these long-lasting beneficial effects were possibly dose dependent [24 h after 0.1 mg/kg, 10.8 ± 0.82% increases F(1,3) = 172.166, P = .0010; 24 h after 0.01 mg/kg, 5.5 ± 2.23% increases, F(1,3) = 12.24, P = .0395]. But performance had returned to baseline level by the sessions conducted 48 h after injection [F(1,3) = 1.47, P = 1.000; F(1,3) = 3.000, P = .1817, respectively]. Huperzine A was tolerated by all the monkeys even at the highest doses. No adverse signs were observed.

Discussion

Scopolamine has been used as a pharmacological tool for understanding pathological impairments such as AD, because it produces amnesiac effects similar to those identified in AD (Sakhakian et al., 1987). In this study, scopolamine dose dependently impaired spatial working memory of young adult monkeys in delayed-response tasks, consistent with the previous reports using other paradigms of delayed-response tasks in monkeys (Rupniak et al., 1989; Ogura and Aigner, 1993), indicating that spatial working memory processes are dependent upon the integrity of the brain cholinergic system. The capacity to perform these tasks requires the bilateral integrity of the dorsolateral prefrontal cortex (PFC) at both short and long delays and the hippocampus mainly at long delays (>15 sec) (Goldman-Rakic, 1987), which receive a massive projection of cholinergic axons originating in basal forebrain. So reduced-choice accuracy caused by scopolamine is due to disruption of the cholinergic system in PFC and the hippocampus through blockade of muscarinic postsynaptic receptors in synaptic clefts. The fact that there was a more significant decrease at longer delays than at shorter delays, although choice accuracy at all retention intervals decreased after scopolamine, showed that the major effects appear to be directly on memory processes.

Huperzine A significantly improved the performance of scopolamine-treated monkeys, producing a bell-shaped dose-response curve, similar to previous findings in rodents (Xiong and Tang, 1995; Cheng et al., 1996). The bell-shaped dose-response curve is common with most drugs that have been reported to exert cognitive enhancing actions; the precise mechanisms of this effect remain to be established. Smaller doses of huperzine A stimulate cognitive function through increasing ACh levels, whereas larger doses mask it via an unknown mechanism. Huperzine A produced AChE inhibition in whole brain or brain regions in a dose-dependent manner following peripheral administration (Tang et al., 1994; Wang and Tang, 1998). Because huperzine A shows no significant affinity for muscarinic receptors (Tang et al., 1989), no evident pre- and postsynaptic effects (Lin et al.,

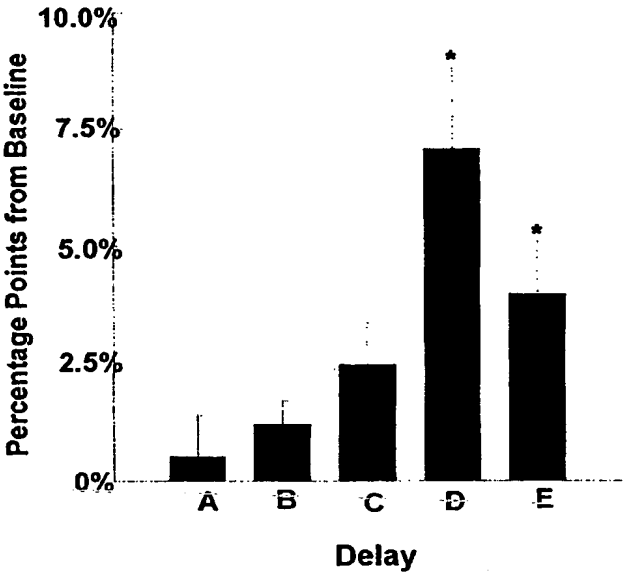


Fig. 6. Effects of huperzine A at the best doses compared with saline control on delayed-response performance for each of the five delayed intervals (A, B, C, D, and E) used in each testing session in aged monkeys (n = 4). The number of trials correct on saline was subtracted from the number of trials correct on huperzine A; this difference score was then multiplied by 3.3% because each trials constituted 3.3% of the total number of trials: [(number correct huperzine A – number correct saline) × 3.3%]. Values in the figure represent mean ± S.E.M. *p < .05 versus saline control.

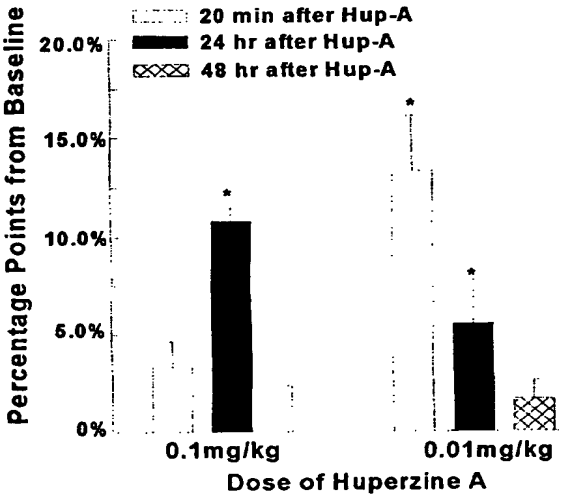


Fig. 7. Effects of huperzine A [0.1–0.01 mg/kg] on delayed-response tasks in aged monkeys (n = 4). Performance was measured 20 min and 24 and 48 h after i.m. injection of huperzine A, respectively. The number of trials correct on saline was subtracted from the number of trials correct on huperzine A; this difference score was then multiplied by 3.3% because each trials constituted 3.3% of the total number of trials: [(number correct huperzine A – number correct saline) × 3.3%]. Values in the figure represent mean ± SEM. *p < .05 versus saline control.

1997), and no effect on ChAT (Tang et al., 1994), its effects on spatial memory in tasks are due primarily to the dose-dependent increase in ACh resulting from direct AChE inhibition. The degree of ACh elevation after huperzine A is selectively highest in frontal and parietal cortex and there are smaller increases in other brain regions (Tang et al., 1989). Considering that ACh is particularly low in the cerebral cortex of AD patients (Bowen et al., 1983), this particularly regional specificity may constitute a therapeutic advantage.

It has been observed that in aged monkeys the number of nicotinic and muscarinic type-1 receptors decreased only slightly with aging, which suggests that postsynaptic indicators of cholinergic function are only mildly impaired with aging. However, the level of ChAT decreased significantly with aging (Wenk et al., 1989). It is mainly because of the loss of cholinergic neurons in forebrain basal nucleus, which projected into the PFC and hippocampus. These age-related changes may underlie a decline in cognitive abilities. Similar to the results found in scopolamine-treated monkeys, the improving efficacy of huperzine A in aged monkeys also exhibited a bell-shaped response. However, the optimal dose is smaller than that for scopolamine-treated monkeys. High-choice accuracy at 0-s delays (about 95%) and unaffected visual discrimination (Bartus and Dean, 1979) shows that the deficits in aged monkeys did not appear to be due to problems with perceptual sensory processing but with memory. The increased ACh concentration induced by huperzine A supplemented the impaired ability of ACh synthesis caused by the decreased level of ChAT, so that the cholinergic transmission is restored close to normal in a certain period. On the other hand, biochemical, electrophysiological, and behavioral studies have indicated an interaction between the cholinergic and noradrenergic systems, as well as the dopaminergic system (Decker and McGaugh, 1991), which influences learning and memory (Kruglikov, 1982). After administration of huperzine A, NE and DA levels were significantly increased over baseline for several hours, whereas ACh levels reach a maximum. These increases in NE and DA levels may be related to the increase of extracellular ACh levels through subcortical mechanism (Zhu and Giacobini, 1995). Therefore, the effects of huperzine A on memory may be associated with the increased levels of NE and DA, which decreased significantly with aging in monkeys (Wenk et al., 1989).

In this study, the performance of all aged subjects after administration of huperzine A was improved. But wide variations in the most-effective dose of huperzine A in aged subjects were observed. This finding is consistent with various levels of loss in the cholinergic system in aged monkeys, suggesting that in clinical therapy the optimal doses of huperzine A must be selected according to pathological situation of the patients with AD. Their overall response as a group, however, was less variable than that of physostigmine-treated aged monkeys in earlier studies (Bartus, 1979), in which some subjects did not benefit from physostigmine at all. The performance of all aged subjects after administration of huperzine A was improved. In the present study, a consistent finding was the long-lasting effect of huperzine A on delayed-response tasks. The performance was significantly different as compared with saline control even 24 h after a single injection of huperzine A (0.1, 0.01 mg/kg) and showed a possible dose-dependent manner. The facts that the terminal half-life of huperzine A was 288 min in humans (Qia et al., 1995), and that huperzine A could produce a long-term inhibition of AChE activity in brain (Wang and Tang, 1998), suggested that the long-lasting effects of huperzine A on cognitive function might simply result from its AChE inhibition.

Compared with rodents, rhesus monkeys may be more

sensitive to pharmacological manipulation of central cholinergic systems (Matsuoka and Aigner, 1997). In addition, primates are able to perform many complex behavioral tasks identical to those impaired in human amnesiac states, including dementia (Freedman and Oscar-Berman, 1986; Sakhakian et al., 1988), in which the delayed-response performance is commonly used to test the mental status of nonhuman primates. Memory-impaired humans show significant performance deficits when tested by this kind of task (Rice, 1987), and the fact that these tasks are sensitive to the impairments associated with human memory loss, supports the validity of using nonhuman primates performing delayed-response tasks as models for development of drugs designed to improve human memory. Huperzine A could improve memory deficits either induced by scopolamine in young adult monkeys or occurring naturally in aged monkeys. These findings extend our previous studies in rodents, in which huperzine A markedly reversed the memory impairments induced by central cholinergic blockade with scopolamine treatment, lesions of the nucleus basalis magnocellularis, or aging (Lu et al., 1988; Cheng et al., 1996; Xiong et al., 1998). Taken together, these results can confirm that huperzine A is a promising candidate for clinical evaluation as treatment for AD.

Acknowledgments

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IN THE MATTER OF
US Serial No: 09/147,490
entitled"Neuroactive Peptide"

EXHIBIT 8

This is Exhibit 8 referred to in Clause 16 of the Statutory Declaration Siew Yeen Chai dated 13th Day of January 2004.

Before me: _____

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BRAIN RESEARCH

Research report

Effects of LY231617 and angiotensin IV on ischemia-induced deficits in circular water maze and passive avoidance performance in rats

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Abstract

The antioxidant LY231617 has previously been shown to offer significant protection against postischemic cell death in the hippocampus and corpus striatum of rats. The present results extend this observation by demonstrating a concomitant protection against the spatial memory deficits that accompany damage to the hippocampus, as measured by the circular water maze task. These animals were further tested for changes in associative memory by employing a passive avoidance conditioning task. No deficits in passive avoidance conditioning were measured among the 4-vessel occlusion animals treated with LY231617 or vehicle. However, the intracerebroventricular injection of angiotensin IV (Ang IV) immediately prior to foot-shock conditioning improved retention of the conditioned response during the subsequent 2-day period. These results suggest that LY231617 can offer considerable protection against global ischemia-induced cell death in the hippocampus with resulting preservation of spatial memory abilities. In addition, untreated animals that suffered cell losses in the hippocampus remained capable of responding to the facilitory effect of centrally administered Ang IV on a non-spatial memory task. The hypothesized mechanisms of the protection characteristics of LY231617, and the nootropic effect of Ang IV, are discussed.

Keywords: Cerebral ischemia; Neuronal death; Hippocampus; Learning; Memory; LY231617; Angiotensin IV; Circular water maze; Passive avoidance conditioning; Rat

1. Introduction

Brain pathology resulting from transient cerebral ischemia due to cardiac arrest, accompanying severe hypotension, and stroke has become a common clinical occurrence that often results in permanent memory deficits [10,17,66,73]. Such impairments can include temporary amnesia involving a loss of memory for recent events, permanently impaired learning and memory, dementias involving attention deficits, and disorders of judgment, reasoning, and spatial orientation. The neurological damage associated with cerebrovascular insufficiency is most frequently located in the medial temporal lobes, the mammillothalamic region, cerebellum, and especially the hippocampus [55,65,67,73]. Considerable research has been directed at gaining an understanding of the mechanisms underlying ischemia-induced brain damage, and has frequently utilized rodent animal models [21]. Whether 4-vessel (4-VO) or bilateral carotid occlusion is employed, damage in the CA1 field is consistently accompanied by deficits in performance of spatial memory tasks [1,69]. Much less damage is reported in the CA3 region and dentate gyrus [3,4,34,58,60]. The CA2 region does reveal some damage, but is not as vulnerable as the CA1 field [1] where cell losses can continue for several days following the ischemic episode [26,35,53].

The mechanisms hypothesized to cause this pathology include excessive release of excitatory amino acids, especially glutamate and aspartate [11,12,22,24,44], glucose deprivation and hypoxia, lactate accumulation and acidosis [51,61], calcium influx [3], and the generation of oxygen-derived free radicals [5,9,27,28,32,49,54,59,62]. Since

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some portion of the ischemia-induced neurological damage is delayed, there is the opportunity for clinical intervention in order to diminish the cell loss and thus decrease the resulting cognitive impairment [13,15,29,33,40,48,52].

Our laboratories have been employing a newly synthesized antioxidant, LY231617, in order to significantly diminish the hippocampal and striatal neuronal cell losses that accompany global ischemia induced by 4-VO [13–15]. To date, no complementary behavioral results have been reported concerning the use of this compound, thus the collection of these data represented a primary purpose of the present investigation.

Along a different direction of research, we have recently discovered a new angiotensin receptor subtype, designated AT_4, that selectively binds the hexapeptide angiotensin IV (Ang IV) and is primarily located on pyramidal cells in the hippocampus, neocortex, basal ganglia, and in the cerebellum [31,45,64]. This AT_4 binding site appears to be involved in memory function [70,71]. The present investigation attempted to bring together these lines of research by testing the notion that since a newly synthesized antioxidant, LY231617, is known to be effective at diminishing cell losses in the hippocampus due to global ischemia, it should also present a savings of memory function in the form of preserving spatial and perhaps conditioned avoidance memory capabilities. We further evaluated the hypothesis that once ischemia-induced cell loss has occurred, the remaining pyramidal cells in the hippocampus can be facilitated via treatment with Ang IV as measured by improved performance on the passive avoidance conditioning task. A preliminary study indicated that Ang IV-induced improvement of performance in the water-maze task was unlikely in animals subjected to global ischemia induced by 4-VO, or those protected from damage with LY231617. The reason for this appears to be a 'behavioral ceiling effect' induced by this task. Since rats do not like the water, but are good swimmers, they expend maximal effort to find the submerged pedestal.

2. Materials and methods

Male Wistar rats (250–320 g, Hilltop Laboratories, Scottdale, PA) were adapted to a 12:12 h light–dark cycle initiated at 07.00 h at 21 ± 1°C for a minimum of 7 days following arrival.

2.1. Global ischemia

The animals were randomly assigned to one of 3 groups: group 1, 4-VO with the antioxidant LY231617 (2,6-bis(1,1-dimethylethyl)-4-[[(1,ethyl)amino]methyl]phenol hydroxochloride]) ($n = 26$)); group 2, 4-VO with vehicle (2% acacia) ($n = 20$); group 3, sham-operated controls ($n = 16$). Members of groups 1 and 2 experienced 30 min of 4-VO according to the methods of Pulsinelli and Brierly [57]. Briefly, each rat was anesthetized with metofane (Pitman-Moore, Inc., Mundelein, IL), the vertebral arteries were occluded by electrocautery, and the common carotid arteries were isolated and atraumatic loops were placed around each without disturbing blood flow. The ends of the loops exited on the ventral surface of the neck. The animals were permitted 24 h of recovery from surgery without food and then, while alert, each rat was subjected to a 30-min period of occlusion by tightening the carotid artery clasps. If the animal did not become unresponsive within 60 s it was removed from further testing. Body temperature was maintained at 37 ± 1°C via an overhead lamp controlled by a rectal thermistor during occlusion and for 30 min thereafter. Blood flow was reinstated by releasing the loops at the end of the 30-min period. The animals in group 1 were treated with LY231617 suspended in 2% aqueous acacia (50 mg/kg orally by gavage) 30 min prior to 4-VO and again 4 h postocclusion. Members of group 2 received only 2% acacia 30 min prior to, and 4 h following 4-VO. Rats in group 3 served as surgical controls. Each animal was anesthetized, and the carotid arteries were isolated as in members of the previous groups; however, loops were not placed around these arteries.

Following 4-VO, the animals were permitted 21 days of recovery and were then tested for motor disturbances that could impair locomotion. Three days following evaluation for motor disturbances the animals were next tested in a circular water maze. Following this testing, each animal was prepared with a chronic intracerebroventricular (i.c.v.) cannula under ketamine hydrochloride (Bristol-Myers, and Co.) accompanied by the muscle relaxant Xylazine (Haver and Co.) (100 and 2 mg/kg i.m., respectively). This procedure has been previously described in detail [72]. Briefly, the guide cannula (PE-60, Clay Adams) was stereotaxically positioned above the right lateral ventricle and fastened in place with 3 skull screws and dental cement. Following a minimum of 48-h recovery, each animal was behaviorally tested for correct cannula placement by the i.c.v. injection of angiotensin II (Ang II, 10 pmol in a total volume of 2 μl of artificial cerebrospinal fluid (aCSF)). This was accomplished by inserting a preloaded 24-gauge stainless steel hypodermic tubing injector into the guide cannula so that it extended 2 mm beyond the tip of the guide, thus penetrating the roof of the lateral ventricle. Ang II was then hand delivered via a 10-μl syringe over a 10-s period. The guide cannula was determined to be correctly placed if a burst of drinking was elicited within 5 min following Ang II injection. Following an additional 48 h of recovery, the animals were tested in the passive avoidance task.

2.2. Behavioral testing

Three behavioral tasks were utilized. The motor function test battery as described by Combs and D'Alecy [16] provided a means of evaluating for differences in motor

abilities among the members of each group following recovery from surgery and ischemic treatment. The circular water maze [46] provided a measure of spatial memory in that animals must locate a submerged pedestal in a pool of water using extra-maze visual location cues. The third task, passive avoidance conditioning, tests associative memory ability in that the animals must associate the dark side of a two compartment apparatus with foot shock and subsequently refrain from entering this compartment.

2.2.1. Motor function test battery

The first test required that each rat be placed on a horizontal screen (62 × 54 cm, grid size 0.6 × 0.6 cm, vertical drop from screen to towels was 1.2 m) that was then rotated into the vertical plane. The duration in s that the animal was capable of holding onto the screen was recorded up to 15 s. The animal received 1 point for 0–5 s, 2 points for 6–10 s, and 3 points for 11–15 s. Following a minimum of 60 min rest period, the animal was next placed on a horizontal wooden dowel (3 cm in diameter × 62 cm long) elevated 1.2 m above the towels. The time that the animal balanced on the rod was measured up to 15 s. The same scoring procedure was used as described above. The third test consisted of timing the duration that the rat could cling to a horizontal hemp rope (1.0 cm diameter) for up to a maximum of 5 s. Points were awarded as follows: 1 point for 0–2 s, 2 points for 3–4 s and 3 points for 4 + s. Each rat could achieve a maximum cumulative score across these 3 tasks of 9 points.

2.2.2. Circular water maze

The water maze consisted of a 1.6-m diameter × 0.6-m tall galvanized cylindrical tank painted black and filled to a depth of 30 cm with 24–26°C water. Three walls of the test room (40 cm from the edge of the tank) were covered with large posters that provided differential spatial cues while the fourth wall was 1.2 m from the edge of the tank thus permitting space for one experimenter to stand. The position of this experimenter was constant following placement of the rat into the maze. A commercially available videotracking system (Chromotrack, San Diego Instruments, San Diego, CA) and accompanying software package was used by a second experimenter (not visible to the animal) that measured each animal's latency and path distance to find the submerged pedestal.

Phase I: acquisition. Acquisition training consisted of 6 days of conditioning with 5 trials per day. Each trial entailed placing the animal into the water facing the wall of the pool within one of 4 quadrants NW, NE, SW, SE, and tracking its swimming path and duration until it found the round submerged pedestal (12 cm diameter, 2 cm below the surface, placed 30 cm from the edge of the tank equidistant from the edges of the quadrant) or 120 s elapsed. If the animal located and mounted the pedestal it was permitted 30 s on the pedestal before the next trial commenced. If the animal did not find the pedestal within 120 s it was placed directly on the pedestal and allowed a 30-s rest period. The entry point and the location of the pedestal were randomly assigned and fixed for each rat prior to acquisition training. At the end of each test day the rat was dried off with a towel and placed under a heat lamp for 10 min before being returned to its home cage.

Phase II: probe trials. On day 7, the pedestal was removed and each rat was placed in the maze at the same entry point as during acquisition training. The times spent immediately at the former location of the pedestal and within the quadrant where the pedestal had been located, were measured for each of 5 trials. The purpose of this phase was two-fold, first it served to determine the persistence of the conditioned response as measured by the time spent precisely at, and within the quadrant where the pedestal had been located. Second, this phase served as extinction trials in preparation for phase III.

Phase III: reversal. On days 9–11, the pedestal was moved to the opposite quadrant from the one used in phase I for each rat. Each rat was placed in the maze at the same location as established in phase I. As in the other phases, one session of 5 trials was conducted for each rat per day.

Phase IV: recall. On day 31 (20 days following reversal training) each rat was tested for an additional 5 trials under the same conditions as those of phase III in order to determine whether the groups differed with respect to recall of reversal training. During the intervening 20 days, the animals were maintained in their home cages without any further testing.

2.2.3. Passive avoidance conditioning

Members of each group were randomly assigned to one of two subgroups designated to receive i.c.v. injected Ang IV, or aCSF, during passive avoidance conditioning. The LY231617-treated animals were assigned such that the Ang IV subgroup contained 14 rats, while the aCSF subgroup consisted of 12 rats. The vehicle-treated group members were assigned to the Ang IV-treated and aCSF-treated subgroups with 10 animals in each group. The sham-operated group was divided such that each subgroup contained 8 rats. The protocol used with this task has been described elsewhere [71]. Briefly, the task required that each animal be habituated to the dark compartment of the apparatus for 5 min with the guillotine door closed. The animal was then returned to its home cage for 5 min and then placed in the lighted compartment (40 W bulb, 40 cm above the compartment) with the guillotine door open. Latency to enter the dark compartment with all 4 feet was measured in s. This was repeated with 5 min in the home cage between trials until the animal entered the dark compartment within 20 s. At this time, the rat received an i.c.v. injection of either Ang IV (1 nmol in 2 μl of aCSF) or aCSF (2 μl) in its home cage as previously described. Following a 5-min delay, the animal was reintroduced into the lighted compartment and permitted to enter the dark compartment. On this last trial, the guillotine door was closed behind the

animal once it entered the dark compartment and the animal received one low-level foot shock (0.2 mA) for 1.5 s via the grid floor. No additional foot shocks were administered to the animal. The rat was then returned to its home cage and placed back into the holding room for 24 h. On days 2–5 each animal was tested for memory retrieval in the passive avoidance apparatus. Each animal was placed in the lighted compartment and latency to enter the dark compartment was measured during one trial per day to a maximum latency of 300 s.

2.3. Histological examination

Following behavioral testing, each rat was deeply anesthetized with equithesin (Jensen-Salsbury Laboratory, 5 ml/kg), intracardially perfused with phosphate-buffered saline, followed by 10% paraformaldehyde. The brains were removed, paraffin-embedded and sectioned at 7 μm through the striatum and hippocampus. The sections were then mounted on slides and stained with hematoxylin and eosin. Damage in the striatum and each field of the hippocampus (CA1–4) was scored by one of the authors (J.A.C.) who routinely performs these evaluations, and was blind to the treatment conditions. Scoring was conducted according to a 0–3 scale: 0, no cell loss; 1, approximately one-third cell loss; 2, approximately two-thirds cell loss; 3, > 90% cell loss.

2.4. Statistical analyses

The data set concerned with the total accumulated score for each animal on the motor function test battery was evaluated using a one-way analysis of variance (ANOVA), for groups. The mean latency and path distance to find the submerged pedestal during each daily block of 5 trials was calculated for each rat for each test day during each phase of the circular water maze protocol. These data sets collected during acquisition training (phase I) were submitted to 3 (Groups) × 6 (Days) ANOVA with repeated measures on the second factor. During phase II, the times spent within the 20-cm-diameter target where the pedestal had been located, and within the appropriate quadrant, were analyzed by one-way ANOVA. The data sets collected during reversal training (phase III) were analyzed by 3 (Groups) × 3 (Days) ANOVA with repeated measures on the second factor. Finally, the data sets generated during recall (phase IV) were analyzed by one-way ANOVA for groups. Neuman–Keuls post-hoc tests were used to further evaluate significant effects with the level of significance set at 0.01. A priori established t-tests were applied to each data set concerned with latency and path distance on day 6 in phase I, and day 11 in phase III. We reasoned that by day 6 of acquisition, and by the third day of reversal training, performance would be stable thus permitting meaningful group comparisons with respect to asymptotic levels.

Latencies by members of the 6 subgroups to enter the dark compartment on the final trial prior to foot shock on day 1 of passive avoidance conditioning were analyzed by a one-way ANOVA. Data concerned with latencies to enter the dark compartment of the passive avoidance chamber on days 2–5 of testing for each of the treatment conditions were each submitted to a 2 (Subgroups) × 4 (Days) ANOVA, with repeated measures on the second factor. Neuman–Keuls post-hoc tests were used to further evaluate significant effects with a level of significance set at 0.01.

Due to the skewed population, the non-parametric Kruskal–Wallis H-test was utilized to compare the rank sums of the damage scores from histological examination of the hippocampus and striatum. Pairwise comparisons were then made using a series of Mann–Whitney U post-hoc tests with the level of significance adjusted to establish the overall type I error probability at α or less.

3. Results

3.1. Histological examination

Of the original 26 animals subjected to 4-VO and treated with LY231617, 6 were discarded because they revealed hippocampal damage scores of 2.6 or greater. We arrived at this cut-off by assuming scores that were 2 standard deviations beyond the mean to be 'statistical outliers' and therefore not properly a member of this treatment population. The behavioral performance of these 6 animals in the circular water maze closely approximated that observed for the 4-VO vehicle-treated group. There were originally 20 rats that experienced 4-VO with vehicle treatment. One of these rats survived this protocol with no measurable damage in the hippocampus or striatum and was removed from this group. This animal's behavioral performance in the circular water maze was excellent, with latencies to find the pedestal of 18, 15 and 10 s on days 4–6, respectively, of acquisition. None of the sham-operated rats exhibited any signs of damage and all were retained. Therefore, the resulting data sets included 20 rats in the 4-VO LY231617-treated group, 19 animals in the 4-VO vehicle-treated group, and 16 rats in the sham-operated control group.

The damage scores ($\bar{x} \pm$ S.D.) measured in the hippocampus for members of the LY231617-treated, vehicle-treated, and sham-operated groups were 0.7 ± 0.9, 2.3 ± 0.7, and 0 ± 0, respectively; and there were differences ($H_2 = 10.74$, $P < 0.05$). Post-hoc analyses indicated that each group was significantly different from the next. Fig. 1 presents representative photomicrographs of the hippocampus from a 4-VO animal treated with LY231617 and a 4-VO vehicle-treated animal. The damage scores seen in the striatum for these same group members were 0.5 ± 0.6, 1.6 ± 0.8, and 0 ± 0, respectively; and again there were

differences ($H_2 = 14.52$, $P < 0.05$). Post-hoc analyses revealed significant differences comparing one group to the next. The damage seen in members of the vehicle-treated group was most intense in the dorsolateral aspect of the striatum.

3.2. Motor functions

Subgroups of animals from the 3 major groups were randomly selected for evaluation. There were no group differences in cumulative scores across the 3 motor tasks, ($F_{2,37} = 1.02$, $P > 0.05$). The mean ($\pm$S.E.M.) for the LY231617-treated ($n = 16$), vehicle-treated ($n = 12$), and sham-operated ($n = 12$) groups were 4.8 (± 0.3), 5.0 (± 0.3) and 5.8 (± 0.3), respectively.

3.3. Behavioral testing

3.3.1. Circular water maze

Fig. 2A shows the mean latencies to find the submerged pedestal for each group during phases I (acquisition), III (reversal training) and retention testing on day 31. In general, the results indicate that those 4-VO animals treated with LY231617 performed equivalently to the sham-operated animals, while the 4-VO rats treated with vehicle performed poorly on this task. Specifically, there were differences in latencies among the groups during acquisition ($F_{2,52} = 10.61$, $P < 0.001$); as expected, there was also a Days effect during acquisition, ($F_{5,260} = 78.16$, $P < 0.001$), indicating improved performance by all groups over days of acquisition training; and there was a signifi-

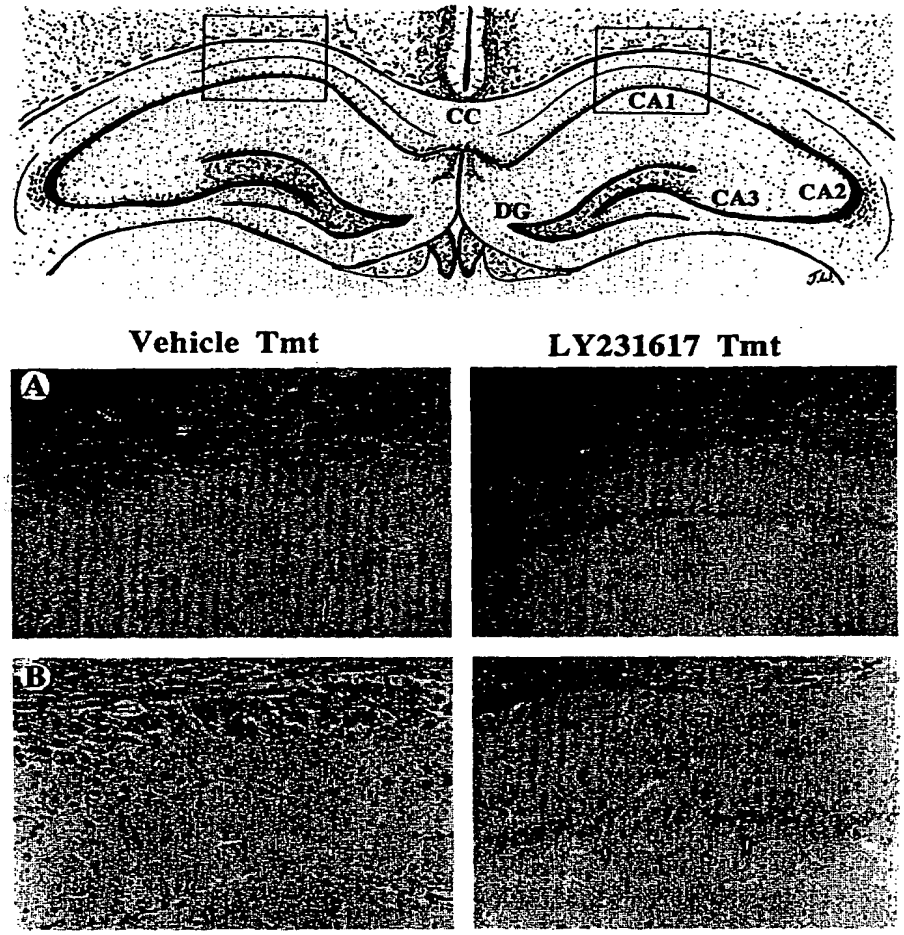

Fig. 1. Photomicrographs of hemotoxylin and eosin-stained sections at the level of the dorsal hippocampus of representative animals treated with 2% acacia and 4-VO (Vehicle Tmt, rat 27) or LY231617 in 2% acacia and 4-VO (LY231617 Tmt, rat 19). A: illustrates the ability of LY231617 treatment to protect hippocampal neurons within the CA1 field (see arrows) from the damage induced by ischemia, compared with the severe loss of these neurons accompanying vehicle treatment and ischemia (×60). B: higher power magnification (×120) of these protected hippocampal neurons in LY231617-treated animals, and the loss of these neurons in vehicle-treated rats. All animals were sacrificed for histological examination between 80 and 100 days following 4-VO. The arrows indicate the degeneration of CA1 pyramidal neurons in the 4-VO vehicle-treated rat and the normal cell density and distribution of pyramidal neurons in the 4-VO LY231617-treated rat. CC, corpus callosum; DG, dentate gyrus.

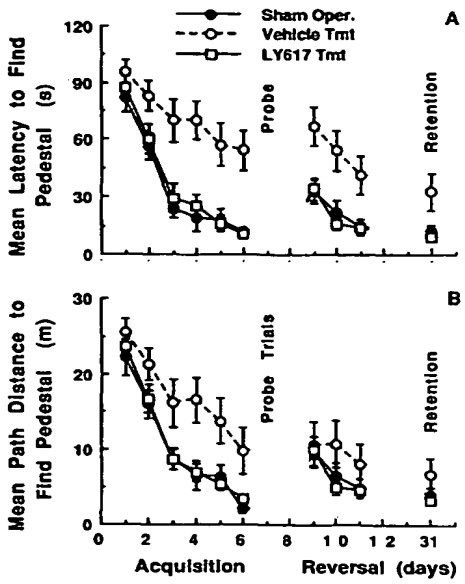

Fig. 2. Changes in mean (±S.E.M.) latencies (A) and swim distances (B) to find the submerged pedestal in the circular water maze during 6 days of acquisition training, 3 days of reversal training with the pedestal relocated to the opposite quadrant, and 1 day of retention testing, for members of the 3 groups. The number of rats in the sham-operated, vehicle-treated, and LY231617-treated groups were: 16, 19, and 20, respectively.

cant interaction effect for Groups × Days ($F_{10,260} = 3.35$, $P < 0.001$). The sham-operated group members, and those 4-VO animals that received LY231617, revealed significantly shorter latencies to find the pedestal than 4-VO rats treated only with vehicle. This difference persisted such that on day 6, the LY231617-treated group displayed the shortest mean latency ($\bar{X} \pm$ S.E.M. = 11.0 ± 1.4 s), followed by the sham-operated animals (12.4 ± 1.8 s), while the vehicle-treated rats required significantly greater time to locate the pedestal (54.8 ± 10.4 s). The sham-operated and vehicle-treated groups were different from one another, as was the comparison of vehicle and LY231617-treated groups, while the sham-operated and LY231617-treated groups did not differ. Probe testing on day 7 (phase II) indicated a pattern of greater time spent in the quadrant where the pedestal was previously located by the LY231617-treated group (44.8 ± 1.8 s), followed by the vehicle-treated rats (36.3 ± 2.9 s), while the sham-operated animals were least persistent (35.8 ± 2.3 s). Fig. 3 represents the time spent within the 12-cm-diameter circle where the pedestal had been located during acquisition training. There were significant differences among the groups ($F_{2,52} = 12.32$, $P < 0.0001$) such that the vehicle-treated animals displayed less time on target (1.0 ± 0.2 s) than the sham-operated (2.1 ± 0.7 s) or LY231617-treated rats (2.2 ± 0.4 s). The two latter groups did not differ. During phase III (reversal training) there were also group differences ($F_{2,52} = 8.19$, $P < 0.001$), a significant Days effect ($F_{2,104} = 24.80$, $P < 0.001$), but no interaction effect. The sham-operated group, and the 4-VO group treated with LY231617, revealed the shortest latencies during reversal training and the vehicle-treated group the longest latencies. By day 11, members of the vehicle-treated group (41.5 ± 10 s) evidenced significantly longer latencies than the sham (14.5 ± 4.1 s) ($t = 4.27$, df = 33, $P < 0.005$), and LY231617-treated groups (13.8 ± 3.0 s) ($t = 3.12$, df = 37, $P < 0.005$). The sham-operated and LY231617-treated groups did not differ. Twenty days after reversal training the animals were tested for retention and there were differences among the groups, ($F_{2,52} = 4.95$, $P < 0.02$). The mean latencies were 12.7 ± 2.9, 9.3 ± 1.3, and 33.1 ± 9.67 s for the sham, LY231617-treated, and vehicle-treated groups, respectively. The vehicle-treated animals revealed significantly slower latencies to find the pedestal than the other two groups that did not differ.

Fig. 2B presents the mean path distances swum in order to locate the pedestal by members of each group during phase I, and there were differences ($F_{2,52} = 6.72$, $P < 0.005$). The 4-VO animals treated with vehicle revealed greater distances than either the sham-operated or 4-VO animals treated with LY231617. There was also a Days effect ($F_{5,260} = 86.92$, $P < 0.001$), and a Groups × Days interaction ($F_{10,260} = 1.96$, $P < 0.05$). On day 6, the vehicle-treated group (9.9 ± 3.1 m) revealed a significantly longer mean swim distance than the sham-operated rats (2.2 ± 0.6 m) ($t_{33} = 4.16$, $P < 0.005$), and also a longer distance than the LY231617-treated group (3.4 ± 0.5 m) ($t_{37} = 3.84$, $P < 0.005$). During probe trials, the LY231617-treated group again showed the greatest mean swim distance in the target quadrant (9.9 ± 0.7 m) in search of the pedestal, followed by the sham-operated rats (9.0 ± 0.5 m), and the vehicle-treated animals (8.1 ± 0.8 m). Reversal training again indicated differences among the groups ($F_{2,52} = 3.62$, $P < 0.05$) with the vehicle-treated rats swimming farther than the other groups; and a significant Days effect, ($F_{2,104} = 29.45$, $P < 0.001$) indicating successful reversal conditioning across groups. There was no Groups × Days interaction. By day 11 the groups had

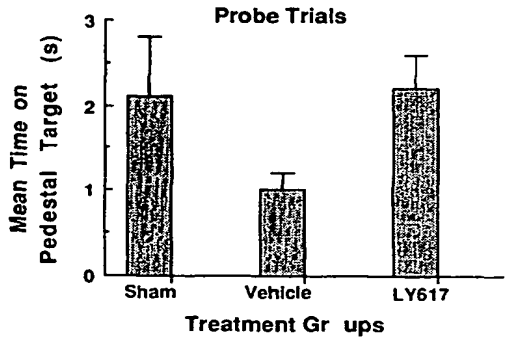

Fig. 3. Mean (±S.E.M.) time within the 12-cm-diameter circle where the pedestal was previously located in the water maze during probe trials on day 7 of testing for members of each group.

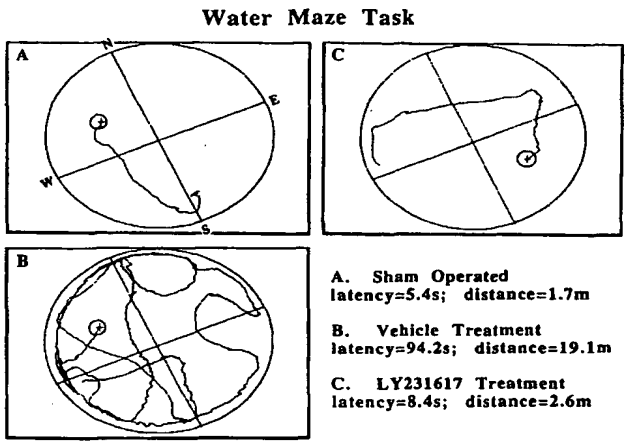

Fig. 4. Representative examples of search patterns in the circular water maze by members of each group on day 6 of acquisition. A: sham-operated, rat 33. B: 4-VO vehicle-treated, rat 14. C: 4-VO LY231617-treated, rat 26.

sorted out such that the LY231617 (4.6 ± 0.9 m) and the sham-operated (4.9 ± 1.3 m) groups revealed significantly shorter mean swim path distances than the vehicle-treated animals (8.2 ± 2.7 m) ($t_{37} = 3.71$ and $t_{33} = 2.07$, respectively, $P < 0.05$). The sham-operated and LY231617-treated groups did not differ. There were no significant group differences during recall testing regarding total distance swum.

Fig. 4 presents representative performance by one animal from each group on day 6 of acquisition. Fig. 4A is the swim pattern evidenced by nearly all of the sham-operated animals, i.e. direct route to the pedestal. Fig. 4B indicates the performance of a 4-VO vehicle-treated rat. This animal was engaged in a search pattern for much of the trial. By this point during training, uncompromised rats confined their search to the target quadrant. Other animals in this group either evidenced this pattern or became fixated and swam along the wall of the maze (thigmotaxis) until the trial ended. Fig. 4C reveals the most frequently observed swim pattern of 4-VO rats treated with LY231617. Often these animals were initially off target, but then quickly reoriented according to the available extra-maze cues and corrected their path to the submerged pedestal.

3.3.2. Passive avoidance conditioning

The results from passive avoidance conditioning are presented in Fig. 5 and indicate that members of each group could satisfactorily acquire the conditioned behavior of avoiding the dark compartment. Further, i.c.v. injection of Ang IV facilitated this conditioned response on the subsequent 2 days of retention testing in the sham-operated and 4-VO rats treated with vehicle. Although a similar trend was present for the 4-VO animals treated with LY231617, there were no statistical differences comparing treated groups ($P = 0.076$). The latencies to enter the dark compartment on the last conditioning trial conducted on day 1 were 5.7 ± 0.7, 7.7 ± 1.2, 4.9 ± 1.0, 5.8 ± 1.0, 6.4 ± 1.0 and 6.1 ± 0.95 s for the sham–Ang IV-, sham–aCSF-, vehicle–Ang IV-, vehicle–aCSF-, LY231617–Ang IV- and LY231617–aCSF-treated subgroups respectively, and these mean latencies did not differ ($F_{5,49} = 0.85$, $P > 0.10$).

Regarding the comparison of sham-operated animals that received an i.c.v. injection of aCSF versus those that received Ang IV (Fig. 5A), there was a difference between subgroups ($F_{1,14} = 5.37$, $P < 0.05$), and a Days effect ($F_{3,42} = 19.55$, $P < 0.001$); however the Groups × Days interaction was not significant. Post-hoc analyses indicated that Ang IV significantly facilitated passive avoidance conditioning on days 2, 3 and 5 of retention testing, but was not different from aCSF injection on day 4. Similar analyses of the 4-VO animals treated with vehicle and then i.c.v. injected with aCSF or Ang IV (Fig. 5B) indicated reasonably comparable results. There was a difference comparing subgroups ($F_{1,17} = 4.43$, $P < 0.05$), a significant days effect ($F_{3,51} = 5.65$, $P < 0.005$), and also a significant Groups × Days interaction ($F_{3,51} = 2.93$, $P < 0.05$). Post-hoc analyses revealed a significant facilitation of conditioning for those rats treated with Ang IV as compared with aCSF, on days 2 and 3, but not on days 4 and 5 of retention testing. Finally, the 4-VO animals

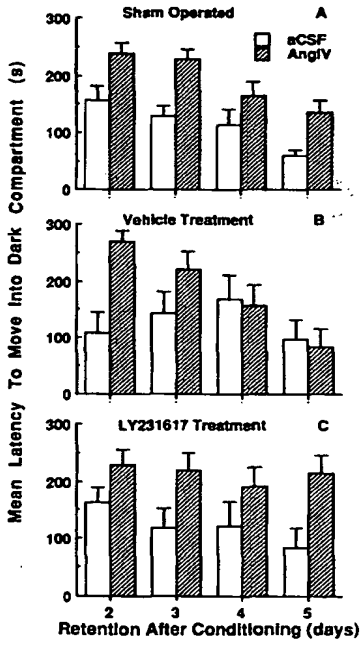

Fig. 5. Mean (±S.E.M.) latencies to move into the dark compartment of the passive avoidance apparatus on days 2–5 of retention testing for members of each group following one foot-shock trial (0.2 mA for 1.5 s) in the dark compartment on day 1 of conditioning. A: sham-operated group. B: 4-VO vehicle-treated group. C: 4-VO LY231617-treated group. Subgroups received an i.c.v. injection of either aCSF (2 μl) or Ang IV (1 nmol in 2 μl aCSF) 5 min prior to the foot-shock conditioning trial.

treated with LY231617 and then administered aCSF or Ang IV in the passive avoidance conditioning task (Fig. 5C) displayed no differences.

4. Discussion

The results of this investigation permit 3 major conclusions. (1) The ischemia-induced damage produced by 4-VO was accompanied by significant impairment in the acquisition of pedestal location in the circular water maze task. Performance during reversal training and 20-day recall test also revealed impairments as compared with sham-operated control animals. (2) 4-VO animals treated with LY231617 revealed significant reductions in the ischemia-induced damage measured in the hippocampus and striatum, and these animals exhibited circular water maze performances that equalled those of the sham-operated controls. (3) 4-VO-induced damage to the hippocampus and striatum did not appear to influence performance on the passive avoidance task, and i.c.v. injection of Ang IV facilitated the conditioned response of avoiding the dark compartment during the initial 2 days of retention testing in the sham and 4-VO vehicle-treated animals.

The 4-VO technique [57] produces reliable neuronal damage in the CA1 field of the hippocampus and the corpus striatum [13,15,58]. This neuronal damage appears to be delayed in its development and is cumulative over several days following the ischemic episode [35,36,58], thus providing an opportunity for clinical interventions designed to protect these most vulnerable cells. Despite considerable research effort, few compounds have been identified that provide such therapeutic protection when tested for efficacy against global cerebral ischemia. The strategy behind the development of these compounds can be traced to the hypothesized mechanism(s) underlying ischemia-induced neuronal damage.

One prominent theory to explain ischemia-induced neuronal cell death concerns phospholipase A_2 (PLA_2), an enzyme that promotes the release of arachidonic acid from membrane phospholipids, thus providing substrate for conversion to peroxides, free radicals, and other products [5,9,32,54]. Free radicals are very reactive in biological systems and usually involve reduction products of oxygen prompting increased levels of toxic intermediates such as hydrogen peroxide, superoxide anion, and hydroxyl radicals. Normally, free radicals are scavenged by various cellular antioxidant defense mechanisms. However, during an ischemic episode with reperfusion, significant elevations in the production of free radicals occur accompanied by a reduced capability of cellular scavenger mechanisms to maintain cellular integrity [14,28,38,62]. Thus, the interaction of hydrogen peroxide and superoxide anion with free or low molecular weight forms of transition metal complexes can yield highly reactive molecules such as hydroxyl free radicals. In turn, free radical-mediated lipid peroxidation can induce cell injury. From these observations, the hypothesis has evolved that free radical scavengers and lipid peroxidation inhibitors could be used to protect against tissue damage following ischemia [7,13,29,30,33,40,63]. The compound used in the present study, LY231617, has previously been shown to inhibit iron-dependent lipid peroxidation in brain homogenates and the neurotoxicity of hydrogen peroxide [15]. This compound provided a significant reduction in CA1 hippocampal and striatal cell death in rats subjected to global ischemia.

The protective effects of this compound are presently extended to include performance on a spatial learning task, the circular water maze. Several advantages of the water maze task over other tests of spatial memory have recently been offered such as dissociation of deficits in memory function from impairment in motivation, sensory and/or motor systems, and memory retrieval mechanisms [43]. Performance on the task has previously been correlated with CA1 hippocampal damage and this task is thought to be the most sensitive behavioral task available to measure such damage [46,47]. The present performance levels of sham-operated and LY231617-treated rats on the circular water-maze task agree with previous reports utilizing rats [6,20,23,42,46]. The deficit in performance seen in the 4-VO rats treated with vehicle were at least as severe as previously published results from hippocampal damaged rats due to ischemia [19] or lesioning [47].

There is on-going debate over the types of memory tasks mediated by the hippocampus [18,41]. If we confine the discussion to those studies that have employed ischemia-induced damage to the dorsal hippocampus via 4-VO or bilateral carotid occlusion, the results indicate deficits in spatial working memory tasks and spatial reference memory tasks [69]. The specific tasks utilized to measure these deficits have included circular water maze, 8-arm radial maze, place discrimination for food, and contextual conditioning. In addition, Wood et al. [69] have recently found deficits in a non-spatial memory task involving object recognition for food. On the other hand, there is no evidence that dorsal hippocampal lesions impair passive avoidance conditioning, although large hippocampal lesions have been reported to increase spontaneous shuttling in a two-way active avoidance task [25], while amygdaloid [39] and dorsolateral striatum lesions [2,37,50,56,68] do impair passive and active avoidance conditioning. Thus, the present results are in general agreement with previous findings in that a significant impairment in the performance of a spatial memory task was observed in those rats that suffered damage to the dorsal hippocampus; however, no impairment in passive avoidance conditioning was observed in the same rats that also presented an ischemia-induced cell loss in the striatum. Presumably, this striatal cell loss was not sufficient to produce an impairment in avoidance conditioning. Comparison of present histological results with previous reports

that verified damage to the striatum, indicated that in those instances of deficits in avoidance conditioning the extent of the striatal damage was significantly greater than presently measured. Our laboratory has recently measured deficits in passive avoidance conditioning in rats prepared with kainic acid-induced bilateral damage to the CA1 or CA3 fields (Stubley-Weatherly et al., submitted). Considerable overlap of CA1 damage was apparent comparing histologies of the present 4-VO vehicle-treated rats, and those lesioned with kainic acid. Resolution of these differences in results will require further research.

Finally, the facilitory effect of i.c.v. injected Ang IV upon passive avoidance conditioning confirms a similar observation from our laboratory [71], and others [8], and extends this finding to include those animals with ischemia-induced cell losses in the hippocampus and dorsolateral striatum. Although these results are of interest, a more clinically relevant question concerns the potential for Ang IV to facilitate acquisition and retention of a spatial memory task in animals that have suffered ischemia-induced hippocampal damage.

The results of the present investigation permit several conclusions: (1) This is the first demonstration that the 'protection' against ischemia-induced neurological damage offered by treatment with the antioxidant LY231617 is accompanied by normal performance on a spatial memory task. (2) There was no measurable impairment in passive avoidance conditioning in 4-VO treated animals that evidenced striatal damage. (3) This study determined that i.c.v. treatment with Ang IV can be utilized to facilitate the retention of passive avoidance conditioning in 4-VO treated rats equivalent with that observed in sham-operated animals.

Acknowledgements

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Contributions of the Brain Angiotensin IV–AT_4 Receptor Subtype System to Spatial Learning

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The development of navigational strategies to solve spatial problems appears to be dependent on an intact hippocampal formation. The circular water maze task requires the animal to use extramaze spatial cues to locate a pedestal positioned just below the surface of the water. Presently, we investigated the role of a recently discovered brain angiotensin receptor subtype (AT_4) in the acquisition of this spatial learning task. The AT_4 receptor subtype is activated by angiotensin IV (AngIV) rather than angiotensins II or III, as documented for the AT_1 and AT_2 receptor subtypes, and is heavily distributed in the CA_1–CA_3 fields of the hippocampus. Chronic intracerebroventricular infusion of a newly synthesized AT_4 agonist (Norleucine^1-AngIV) via osmotic pump facilitated the rate of acquisition to solve this task, whereas treatment with an AT_4 receptor antagonist (Divalinal) significantly interfered with the acquisition of successful search strategies. Animals prepared with bilateral knife cuts of the perforant path, a major afferent hippocampal fiber bundle originating in the entorhinal cortex, displayed deficits in solving this task. This performance deficit could be reversed with acute intracerebroventricular infusion of a second AT_4 receptor agonist (Norleucinal). These results suggest that the brain AngIV–AT_4 system plays a role in the formation of spatial search strategies and memories. Further, application of an AT_4 receptor agonist compensated for spatial memory deficits in performance accompanying perforant path knife cuts. Possible mechanisms underlying this compensatory effect are discussed.

Key words: spatial memory; hippocampus; perforant path knife cuts; angiotensin IV analogs; AT_4 receptor; circular water maze

Several classic roles have been ascribed to the brain renin angiotensin system, including blood pressure regulation, body fluid homeostasis, cyclicity of reproductive hormones and sexual behavior, and regulation of pituitary hormones (for review, see Johnston, 1990; Saavedra, 1992; Wright and Harding, 1992; Fitzsimons, 1998). These functions are mediated by the angiotensin receptor subtype AT_1, with less involvement by the AT_2 subtype. Both of these receptor subtypes are activated by the octapeptide angiotensin II (AngII) and the heptapeptide angiotensin III (AngIII) (for review, see Smith, 1996; Wright and Harding, 1997). Recently, our laboratory discovered and characterized a third angiotensin binding site (Harding et al., 1992; Swanson et al., 1992; Zhang et al., 1999), designated AT_4 (de Gasparo et al., 1995). This receptor subtype is activated by the hexapeptide angiotensin IV (AngIV) and is prominent in brain structures important to cognitive processing and sensorimotor functions, including neocortex, hippocampus, dentate gyrus, thalamus, and cerebellum (Miller-Wing et al., 1993; Wright et al., 1995). The AT_4 subtype is also present in the nucleus basalis magnocellularis (NBM) and medial septum in high densities (Møeller et al., 1996). In contrast, AT_1 and AT_2 subtypes are poorly represented in the neocortex, hippocampus, dentate gyrus, and cerebellum (Wright et al., 1995; Wright and Harding, 1997), although a recent immunohistochemical study has reported AT_1-positive immunoreactivity in the dentate gyrus and subiculum, with lesser staining in the CA_3 field and few stained cells observed in CA_1 and CA_2 fields (von Bohlen und Halbach and Albrecht, 1998). Intracerebroventricular injection of AngIV has been shown to stimulate c-fos expression in the CA_1–CA_3 fields of the hippocampus (Roberts et al., 1995) and to facilitate passive-avoidance conditioning in rats (Braszko et al., 1988; Wright et al., 1993). These results suggest that intracerebroventricular delivery of this peptide influences the hippocampus.

The notion that the hippocampus plays an important role in spatial memory processing is supported by the observation that damage to the hippocampus results in impaired ability to solve tasks that rely on spatial search strategies (Olton et al., 1978; Morris et al., 1990; Sutherland and McDonald, 1990). Thus, hippocampal damage has been correlated with disruption of spatial memory in a number of mammalian species, including rat (Morris et al., 1982; Sutherland et al., 1982, 1983; Nadel, 1991; Jarrard, 1993) and human (Volpe and Hirst, 1983; Cummings et al., 1984; Zola-Morgan et al., 1986). Bilateral hippocampal lesions have also been shown to interfere with acquisition and/or retention of spatial memory in rats as measured by performance on the circular water maze task (Morris et al., 1982; Rudy and Sutherland, 1989; Stubley-Weatherly, 1996).

Our laboratory has synthesized and characterized several AT_4-specific agonists, including Norleucine^1-AngIV (Nle^1-AngIV), Norleucinal (Sardinia et al., 1994; Wright et al., 1995), and AT_4

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Table 1. Structure, molecular weight, peptide content, AT_4 binding affinity, and source of each peptide examined

Compound	Structure	Molecular weight	Content % weight	k_i(M)[a]	Source
AngII(4–8)	Tyr-Ile-His-Pro-Phe	675	70	$>1 \times 10^{-6}$	Peninsula Labs (catalog #7037)
AngIV	Val-Tyr-Ile-His-Pro-Phe	774	73	$2.63 \pm 0.12 \times 10^{-9}$	Peninsula Labs (catalog #7025)
Nle[1]-AngIV	Nle-Tyr-Ile-His-Pro-Phe	788	72	$3.59 \pm 0.51 \times 10^{-12}$	Harding, Hanesworth
Norleucinal[b]	Nle ψ Tyr-Ile-His-Pro-Phe	770	72	$1.80 \pm 0.20 \times 10^{-10}$	Harding, Hanesworth
Divalinal[b]	Val ψ Tyr Val ψ-His-Pro-Phe	734	70	$1.29 \pm 0.35 \times 10^{-10}$	Harding, Hanesworth

[a]Binding affinities from Sardinia et al. (1993, 1994), Wright et al. (1995), and Krebs et al. (1996) using heat-treated bovine adrenal membranes.
[b]ψ, CH_2-NH bond.

receptor antagonists, including Divalinal (Krebs et al., 1996). The present investigation evaluated the efficacy of Nle[1]-AngIV to facilitate the acquisition of the circular water maze task and of Divalinal to disrupt acquisition. We also determined whether Norleucinal treatment compensated for deficits in spatial memory produced by damage to the perforant path (PP). Norleucinal was used rather than Nle[1]-AngIV because of its added resistance to degradation. The PP is the major afferent pathway to the hippocampus projecting from the entorhinal cortex to the dentate gyrus, passing near the dorsal hippocampal commissure (Hjorth-Simonsen and Jeune; 1972; Skelton and McNamara, 1992; Klug et al., 1998).

MATERIALS AND METHODS

Subjects

Male Sprague Dawley rats (270–350 gm, Charles River-derived) were adapted to a 12 hr light/dark cycle initiated at 7:00 A.M. in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) approved vivarium at a temperature of 21 ± 1°C. The animals were housed singly and were permitted access to water and Purina laboratory rat chow *ad libitum*; however, food was removed the night before surgery.

Apparatus

Motor test battery. All animals were tested for potential treatment-induced disturbance in motor ability using a motor test battery developed by Combs and D'Alecy (1987). The first test of this battery required that each animal be placed on a horizontal screen (62 × 54 cm, grid size of 0.6 × 0.6 cm) that was rotated into the vertical plane with a vertical drop from the screen to towels of 1.2 m. The duration in seconds that the animal was capable of holding onto the screen was recorded up to a maximum of 15 sec. The animal received one point for 0–5 sec, two points for 6–10 sec, and three points for 11–15 sec. After a 20 min rest period, the animal was next placed on a horizontal wooden dowel (3 cm in diameter × 62 cm long), also elevated 1.2 m above the towels. The time that the animal balanced on the rod was measured up to 15 sec. The same scoring procedure was used as described above. The third test consisted of timing the duration that the animal could cling to a horizontal hemp rope (1.0 cm diameter) using its forearms for up to a maximum of 5 sec. The vertical drop onto towels was 1.0 m. Points were awarded as follows: one point for 0–2 sec, two points for 3–4 sec, and three points for >4 sec. Each animal could achieve a maximum cumulative score across the three tasks of nine points.

Circular water maze. The circular water maze task developed by Morris (1981, 1984) was used to test spatial learning ability. The water maze consisted of a 1.6 m diameter × 0.6 m tall galvanized cylindrical tank painted black and filled to a depth of 30 cm with 26–28°C water. Three walls of the test room (40 cm from the edge of the tank) were prepared with visual cues consisting of circles on one wall, triangles on a second wall, and squares on a third wall. The fourth wall was 1.2 m from the edge of the tank, thus permitting space for one experimenter to stand. The position of this experimenter was constant after placement of the animal into the maze. A commercially available video tracking system and accompanying software package (Chromatrak; San Diego Instruments, San Diego, CA) was used to measure each animal's latency and path distance to find the submerged pedestal.

The animals used in the first two experiments concerned with chronic treatment of Nle[1]-AngIV and Divalinal were prepared with osmotic pumps and, after 24 hr of recovery acquisition training was initiated consisting of five trials per day for 6 d. At the completion of this phase, one probe trial (description follows) was conducted, each osmotic pump was then removed under local anesthesia (Lidocaine; Elkins-Sinn, Cherry Hill, NJ), and the wound was closed with skin staples (model 8024–12; Davis and Geck, American Cyanamid, Wayne, NJ). After 1 d of recovery, 6 additional training days were conducted to determine whether treatment-induced deviations in acquisition could be overcome after termination of drug delivery. During these additional training days, the submerged pedestal was positioned in the opposite quadrant to the initial placement for each rat. At the completion of this phase, one additional probe trial was conducted.

Members of a third experiment were prepared with bilateral perforant path knife cuts and similarly tested; however, no osmotic pumps were implanted, rather acute intracerebroventricular injections were administered as described below. These animals received 8 d of acquisition training, five trials per day, with one probe trial conducted at the completion of testing on day 8.

Each trial entailed placing the animal into the water facing the wall of the pool at one of four locations [north (N), south (S), east (E), and west (W)] and tracking its swimming path and duration until it found the round submerged pedestal (12 cm diameter painted black, 3 cm below the surface). The pedestal was placed 30 cm from the edge of the tank equidistant from the edge of the quadrant within one of the four quadrants: NW, NE, SW, and SE. If the animal located and mounted the pedestal, it was permitted 30 sec on the pedestal before the next trial commenced. If the animal did not find the pedestal within 120 sec, it was placed directly on the pedestal and allowed a 30 sec rest period. The animal's entry point was randomized on each trial, although the location of the pedestal was initially randomly assigned but remained fixed for each animal during acquisition training, and was repositioned as described above after pump removal for members of experiments 1 and 2. At the end of each test day, the animal was dried off with a towel and placed under a 100 W lamp for 10 min before being returned to its home cage. After the fifth trial of days 6 and 13, the pedestal was removed for members of the first two experiments, and a 2 min probe trial was completed. The animals of the third experiment were administered one probe trial at the conclusion of testing on day 8. During these probe trials, the time spent within the quadrant, as well as the number of crossings into and out of the quadrant where the pedestal had been located, were recorded.

Compounds. Table 1 provides information on the structures of the compounds, their molecular weights, peptide content as determined by HPLC analyses, AT_4 receptor binding affinity, and the source, i.e., synthesized in our laboratory or provided by a commercial supplier. An automated peptide synthesizer (Coupler 250; DuPont Wilmington, DE) was used to prepare peptides not commercially available. Angiotensin IV is included for reference only. Peptide purity ranged from 90–100%, whereas acetate represented the major contributor to the decreased peptide content. Corrections were made for differences in peptide content and purity when the compounds were prepared for use.

Design and procedures

Nle[1]-AngIV treatment. Twenty-four rats were randomly assigned to one of three treatment groups ($n = 6$ each) or an artificial CSF (aCSF) control group ($n = 6$). Each rat was anesthetized with ketamine hydrochloride (100 mg/kg, i.m.; Bristol-Myers, Syracuse, NY) and xylazine (2

mg/kg, i.m.; Haver-Mobey, Shawnee, KS) and prepared with a 7 d osmotic pump (model 2001; Alza Scientific Products, Palo Alto, CA) that infused intracerebroventricularly at the rate of 1 μl/hr. This was accomplished via a stereotaxically positioned length of hypodermic stainless steel tubing (23 ga, length of 3.2 cm) prepared with a 90° bend such that a 7 mm length of the tubing was inserted through a skull trephine hole, thus penetrating the roof of the lateral ventricle. Flat-skull coordinates used for placement of the trephine hole were 1.0 mm posterior (P) to bregma and 1.5 mm lateral (L) to midline (Paxinos and Watson, 1986). The stainless steel tubing was anchored to the cranium with stainless steel screws and dental cement. The pump was connected to the stainless steel tubing via PE-60 tubing (Clay Adams, Parsippany, NJ) and placed subcutaneously between the scapulas. These animals were given 1 d to recover and then testing was initiated, first on the motor function test battery and then daily in the circular water maze.

Members of groups 1, 2, and 3 received 0, 0.1, and 0.5 nmol/hr, respectively, the AT_4 receptor agonist Nle^1- AngIV. The 0 nmol/hr group represented an aCSF infusion control group. These doses of Nle^1-AngIV were established in a preliminary investigation that noted a significant improvement in acquisition with intracerebroventricular administration of 0.1 and 0.5 nmol/hr compared with controls but no additional facilitation of acquisition when comparing the 0.5 and 1.0 nmol/hr doses. Members of group 4 received the pentapeptide AngII(4–8) (catalog #7037; Peninsula Laboratories, Belmont, CA) at a dose of 0.5 nmol/hr. Angiotensin II(4–8) has been shown to bind with low affinity at the AT_4 receptor site (Sardinia et al., 1993) (Table 1). After the completion of day 6 of acquisition trials, each animal was tested for strength of conditioning using a probe trial. After a 2 hr rest period, each animal was further evaluated for sensorimotor deficits by using a visible pedestal (2 cm above the surface of the water). Both the entry points (N, S, E, W) and locations of the visible pedestal (NW, NE, SW, SE) were randomly assigned for each rat on each of five trials. In all other respects, these trials were conducted as described above. The osmotic pumps were removed on day 7, and acquisition trials were resumed beginning on day 8 for an additional 6 d, with the pedestal repositioned to the opposite quadrant for each animal. On day 13, an additional probe trial was conducted.

Divalinal treatment. Twenty-four rats were randomly assigned to a nontreated control group ($n = 6$) or one of three treatment groups ($n = 6$ each): 0, 0.5, or 5.0 nmol/hr Divalinal. These doses of Divalinal were determined in a preliminary study that noted no differences in inhibition of acquisition when comparing 5.0 and 10.0 nmol/hr. Each member of the treatment groups was prepared with a 7 d osmotic pump as described above. Training trials, probe trials, and the visible pedestal protocols were identical with the procedures described in the first experiment.

Perforant path knife cuts. Thirty-two rats were randomly divided between two major groups (16 rats each) and were prepared as follows during one surgical session under ketamine hydrochloride and xylazine. Members of the first group received bilateral PP knife cuts directed at the medial and lateral perforant path tracts that extend from the entorhinal cortex to the dentate gyrus, according to the protocol offered by Skelton and McNamara (1992). These knife cuts were accomplished in two steps using a stereotaxically held knife blade (width of 7 mm, thickness of 0.2 mm; Fine Science Tools, Foster City, CA) with flat-skull coordinates relative to bregma of P, 8.0; L, 3.5; and V, 6.0 mm from dura. From this starting point, the knife was moved medially 0.5 mm. The blade was removed and repositioned according to the following coordinates: P, 8.0; L, 4.8 and V, 6.5 mm from dura. From this starting point, the blade was again moved medially 0.5 mm and withdrawn. This procedure was then repeated in the opposite hemisphere. The animals of the second group served as surgical controls and received equivalent knife cuts of the occipital cortex directly superior to the location of the perforant path cuts made in members of the first group. These neocortex knife cuts were made according to the same posterior and lateral coordinates; however, the blade was lowered only 3 mm V to dura. Each animal was also prepared with an intracerebroventricular guide cannula (PE-60) stereotaxically positioned above the right lateral ventricle and fastened in place with skull screws and dental cement. This procedure is similar to that described above and has been reported previously in detail (Wright et al., 1985).

After 7 d of recovery, each animal was behaviorally tested for correct cannula placement by the intracerebroventricular injection of AngII (10 pmol in 2 μl of aCSF). This was accomplished by inserting a preloaded 30 ga stainless steel hypodermic tubing injector, prepared with a 24 ga stainless steel tubing sleeve, into the guide cannula such that it extended 2 mm beyond the tip of the guide, thus penetrating the roof of the lateral ventricle. Angiotensin II was then hand delivered via a 10 μl Hamilton syringe over a 30 sec period. The guide cannula was considered to be correctly placed if a burst of drinking was elicited within 5 min after AngII injection. After an additional 2 d of recovery, the animals were divided into subgroups ($n = 8$ rats each), which were treated with intracerebroventricular bolus injections of Norleucinal (1.0 nmol in 2.5 μl aCSF) or aCSF (2.5 μl) 5 min before testing for motor dysfunction using the motor function test battery. This was followed by 8 d of acquisition training in the circular water maze task, also preceded by the intracerebroventricular injection of Norleucinal or aCSF 5 min before training. At the completion of testing on the final day, a probe trial was conducted for each animal as described above.

Histology

Correct placement of the intracerebroventricular guide cannula for each animal used in the first two experiments was confirmed by the intracerebroventricular injection of 10–12 μl of fast green dye via the chronic cannula under equithesin anesthesia (3.5 ml/kg, i.p.; Jensen-Salsbury Laboratory, Kansas City, MO), followed by brain extraction and visual confirmation of dye within the brain ventricles. Each osmotic pump was also checked to see whether its contents had been exhausted.

After behavioral testing, each animal used in the third experiment was deeply anesthetized with equithesin and intracardially perfused with PBS, followed by 10% paraformaldehyde. The brains were removed and stored in 10% formaline and, 48 hr before sectioning, the brains were transferred to a 10% formaline–20% sucrose solution. Each brain was sectioned at 14 μm in the horizontal plane using a cryostat (Jung frigocut 2800E; Leica Instruments, Nussloch, Germany). Every third section through the knife cut was mounted on electrostatic microscope slides (Fisher Scientific, Pittsburgh, PA) and stained via a modified hematoxylin and eosin staining technique for verification of damage. The modified staining procedure consisted of the following sequence: (1) Gill-2 hematoxylin (2 min); (2) dH_2O (10 dips); (3) acid rinse [2 ml of 0.2% HCl–1 dH_2O (10 dips)]; (4) dH_2O (10 dips); (5) bluing reagent [2 ml of 30% $NH_4(OH)$–1 dH_2O (1 min)]; (6) $dH_2O \times 2$ (10 dips each); (7) 95% EtOH × 2 (10 dips each); (8) eosin-Y (1 min); (9) 95% EtOH (10 dips); (10) 95% EtOH (10 dips); (11) 100% EtOH × 3 (10 dips each); and (12) xylene × 3 (10 dips each).

Knife cut damage was quantified by a computerized scanning device (Envisions Scanner, Trans env. 24 pro; International Business Machines, White Plains, NY) connected to an IBM personal computer formatted with appropriate software (Adobe Photoshop, Adobe Systems, San Jose, CA). After each initial scan, the area of damage was calculated via an additional computer package (SigmaScan Image; Statistical Program for the Social Sciences, Chicago, IL) that provided lesion size, as well as overall structural region size, in square millimeters. Reconstruction of the lesion along the dorsoventral plane permitted calculation of lesion volume in cubic millimeters. The knife cut damage was then converted to percent of total perforant path volume for each hemisphere. Hjorth-Simonsen and Jeune (1972) and Paxinos and Watson (1986) were consulted regarding identification of structures damaged.

Data analyses

The data sets concerned with the total score for each animal on the motor function test battery were evaluated using a one-way ANOVA. Significant effects were further evaluated using Newman–Keuls *post hoc* tests with a level of significance set at $p = 0.01$.

The mean latency and path distance to find the submerged pedestal during each daily block of five trials was calculated for each animal for each day of acquisition. Because we anticipated group differences only during early training trials in the first experiment, these data were submitted to *a priori* established separate one-way ANOVAs for days 1, 6, and 13 of testing. Given the use of an AT_4 receptor antagonist in experiment 2, we predicted significant interference with acquisition during the entire 6 d treatment period. Therefore, these data were submitted to a groups × days ANOVA, with repeated measures on the second factor. Once again, *a priori* established one-way ANOVAs were applied to each data set concerned with latency and path distance on days 1, 6, and 13 of acquisition. We reasoned that, by the final day of acquisition, performance would be reasonably stable, thus permitting meaningful group comparisons regarding asymptotic levels. Similarly, one-way ANOVAs were used to determine whether there were differences among groups during probe trials and during the visible pedestal protocol. In the third experiment, we expected group differences to

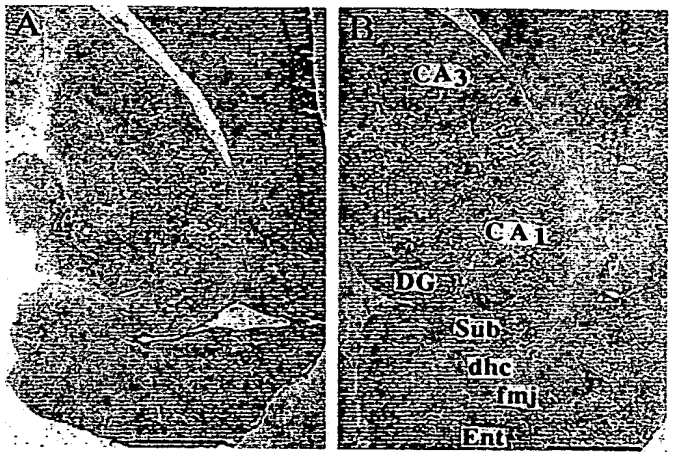

Figure 1. Representative photomicrographs of perforant path knife cuts (*A*) and control knife cuts to the neocortex (*B*) from animals treated with Norleucinal. Horizontal sections of the right hemisphere were taken at the level of 4.1 mm ventral to bregma according to Paxinos and Watson (1986). These knife cuts transsected the perforant pathway at the anteroposterior level of the dorsal hippocampus commissure (*dhc*), forceps major of the corpus collosum (*fmj*), and subiculum (*Sub*). These cuts isolated the entorhinal cortex (*Ent*) from the dentate gyrus (*DG*) and other anterior brain structures, such as the CA_1–CA_3 fields. Scale bar, 1 mm.

persist over all 8 d of acquisition training. Therefore, these data were submitted to a groups × days ANOVA, with repeated measures on the second factor. A one-way ANOVA was used to test for group differences during probe trials.

RESULTS

Histological findings

All animals of the first two experiments were found to have correctly placed intracerebroventricular guide cannulas, as evidenced by the presence of dye within the lateral ventricles. Each osmotic pump was also found to be either empty or nearly empty. The animals of the third experiment that received PP knife cuts and were treated with Norleucinal exhibited an overall mean ± SEM of 66.7 ± 3.2% damage to this pathway (left hemisphere, 67.0%; right hemisphere, 66.3%). Those animals prepared with PP knife cuts and treated with aCSF revealed an overall mean ± SEM of 64.4 ± 4.2% damage (left hemisphere, 65.3%; right hemisphere, 63.5%). Representative photomicrographs are presented from one animal of the PP path knife cut group treated with Norleucinal (Fig. 1*A*) and one animal from the neocortex knife cut group treated with Norleucinal (Fig. 1*B*). These knife cuts were discrete and transsected the PP at the anteroposterior level of the dorsal hippocampal commissure and subiculum, thus isolating the entorhinal cortex from more anterior brain structures. In all rats, the knife cuts passed through the forceps major of the corpus callosum, and the dorsoventral extent of damage generally spanned the full range of entorhinal cortices. There was no discernable damage noted in the dentate gyrus or CA_1–CA_3 fields of the hippocampus. The knife cut control rats evidenced damage to the neocortex overlying the PP–entorhinal cortex region; however, these latter structures remained intact. Members of the neocortex knife cut control groups that received Norleucinal or aCSF revealed no loss of PP fibers.

Motor and sensory functions

There were no differences in cumulative scores across the three motor tasks comparing the groups of the first experiment ($F_{(3,20)} = 2.78$; $p > 0.05$). The mean ± SEM scores for the groups treated with 0.5, 0.1, and 0 of nmol Nle^1-AngIV, and 0.5 nmol of pentapeptide were 8.4 ± 0.2, 8.2 ± 0.3, 7.3 ± 0.4, and 8.4 ± 0.2, respectively. There were also no differences among the groups used in the second experiment ($F_{(3,20)} = 1.84$; $p > 0.10$). The mean ± SEM scores for the groups treated with 5.0, 0.5, and 0 nmol/hr Divalinal and nontreated controls were 7.0 ± 0.2, 6.3 ± 0.3, 6.3 ± 0.3, and 7.0 ± 0.2, respectively. Comparable scores from the third experiment were as follows: PP cut group, 6.9 ± 0.5; cortex control knife cut group, 7.4 ± 0.4. These groups did not differ ($F_{(1,30)} = 1.12$; $p > 0.10$). Thus, there was no evidence of motor dysfunction among any of the animals used in these experiments.

Similar results were noted for the visible pedestal trials. Specifically, no differences among the groups were measured in the first experiment with respect to latency to find the visible platform ($F_{(3,20)} = 1.10$; $p > 0.10$) or distance swam to locate the pedestal ($F_{(3,20)} = 0.87$; $p > 0.10$). Similar findings were noted for the groups of the second and third experiments, i.e., no differences concerning latency or distance swam to find the visible pedestal.

Circular water maze

Nle^1-AngIV treatment

Figure 2*A* presents the mean ± SEM latency to find the submerged pedestal for each group during the initial 6 d of testing with the osmotic pumps in place and during the subsequent 6 d after pump removal. Figure 2*B* presents the mean ± SEM path distances swam by members of each group. Overall, the results indicate that those animals treated with Nle^1-AngIV performed better on the initial 2 d of acquisition, with respect to latency and distance swam to find the pedestal, than members of the control group infused with aCSF or those rats infused with the pentapeptide. Figure 3 provides representative examples of performance by an animal treated with 0.5 nmol/hr Nle^1-AngIV on days 1 and 6 of acquisition compared with an animal infused with 0.5 nmol/hr pentapeptide. The Nle^1-AngIV-treated animal evidenced a superior search strategy on day 1 compared with the rat infused with pentapeptide. Generally, members of the Nle^1-AngIV-treated groups appeared to make use of extramaze spatial cues to locate the pedestal earlier in their acquisition trials compared with members of the pentapeptide- and aCSF-infused groups. Thus, their search patterns progressed to more productive strategies sooner than members of the other two groups. However, by day 6 of acquisition, the performances of these animals were equivalent. After pump removal, members of all four groups performed nearly equivalently, although the pentapeptide-treated rats evidenced slightly poorer performance than members of the other groups on days 9–11.

The statistical analyses to support these conclusions indicated a significant groups effect concerning latency to find the pedestal on day 1 ($F_{(3,20)} = 6.08$; $p < 0.005$). *Post hoc* analyses revealed that those rats infused with 0.5 nmol/hr Nle^1-AngIV revealed significantly shorter latencies to find the pedestal than the aCSF-infused rats or those rats treated with pentapeptide. The animals infused with 0.1 nmol/hr Nle^1-AngIV displayed shorter latencies than the aCSF-infused animals but were not different from the pentapeptide group or those rats infused with 0.5 nmol of Nle^1-AngIV. A similar pattern emerged for path distance, with an overall difference among groups on day 1 of acquisition ($F_{(3,20)} = 3.18$; $p < 0.05$). Both groups treated with Nle^1-AngIV evidenced shorter path distances than those animals infused with aCSF or

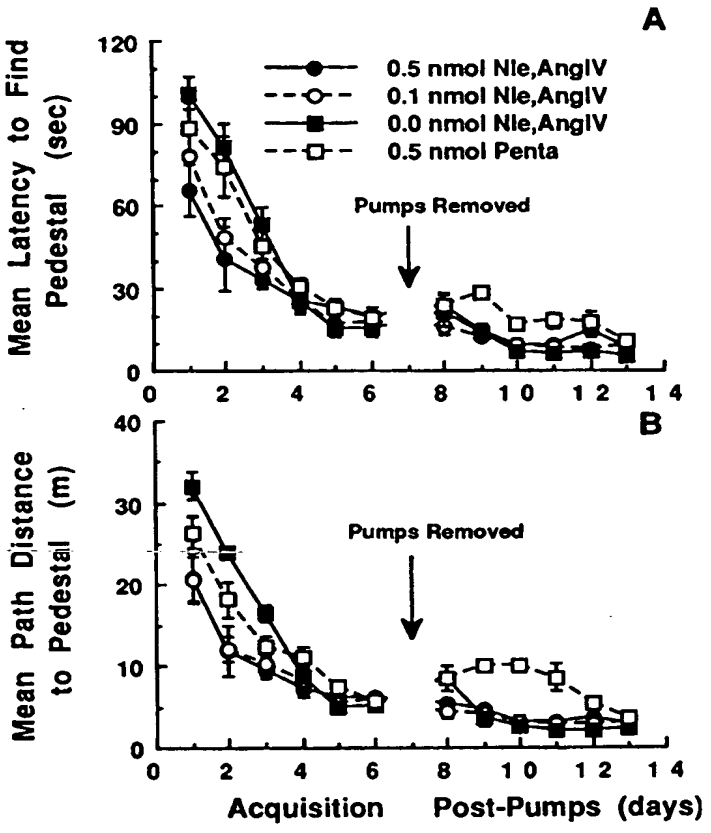

Figure 2. Mean ± SEM group changes in latencies (*A*) and swim distances (*B*) to locate the submerged pedestal in a circular water maze task during 6 acquisition days with osmotic pumps in place, followed by 6 additional acquisition days with the pumps removed. All treatments were intracerebroventricularly delivered via osmotic pump at the indicated doses in a volume of 1 μl aCSF/hr. Those animals treated with 0.5 nmol of Nle^1-AngIV performed better during days 1 and 2 of acquisition with respect to latency to find the pedestal ($p < 0.005$) and swim distance ($p < 0.05$) than members of the control group (0.0 nmol of Nle^1-AngIV) or those rats infused with AngII(4–8) (*Penta*). Pentapeptide binds at the AT_4 receptor with low affinity. These groups did not differ during days 3–6 of acquisition. After pump removal, the location of the submerged pedestal was shifted to the opposite quadrant for each animal. Although those animals treated with pentapeptide revealed longer swim distances to find the pedestal on days 9–11, by days 12 and 13, the groups did not differ. Each group consisted of six rats surgically prepared with a 7 d osmotic pump and were given 1 d to recover before the initiation of acquisition trials. Five trials were administered per day with entry points randomly assigned (N, S, E, W), although the location of the submerged pedestal was fixed for each rat.

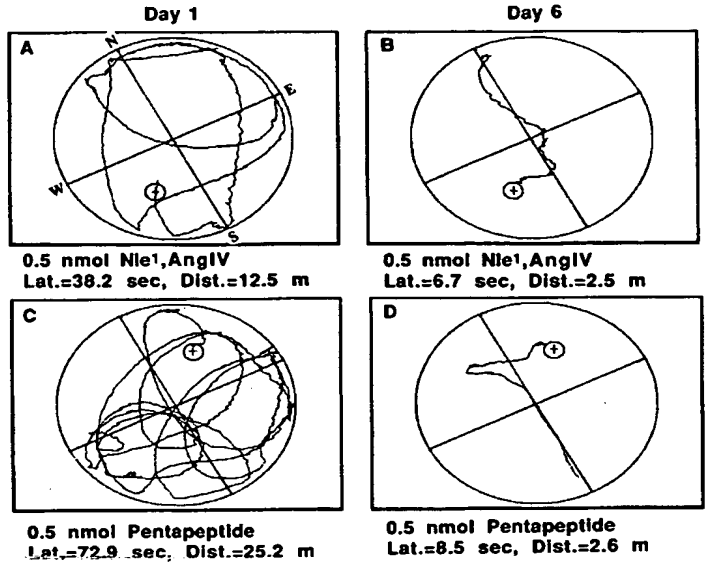

Figure 3. Representative examples of search patterns in the circular water maze during a 2 min trial by one member of the group treated intracerebroventricularly with 0.5 nmol/hr Nle^1-AngIV during days 1 (*A*) and 6 (*B*) of acquisition training and one member of the group treated with 0.5 nmol/hr pentapeptide during days 1 (*C*) and 6 (*D*) of acquisition. Latency (*Lat*) in seconds to find the submerged pedestal and distance swam (*Dist*) in meters are indicated for each animal. Those rats treated with Nle^1-AngIV displayed a superior search strategy compared with animals treated with AngII(4–8) (*Pentapeptide*) on day 1 of acquisition, as evidenced by significantly shorter latencies ($p < 0.05$) and swim distances ($p < 0.05$) to find the submerged pedestal. By day 6, all animals had acquired efficient search strategies and did not differ. Each group consisted of six rats prepared with 7 d osmotic pumps that infused at a rate of 1 μl/hr aCSF.

pentapeptide. By day 6 of acquisition, no differences among groups were seen concerning latency or path distance to locate the pedestal. This was also true at day 13 after pump removal.

The results of probe trials conducted on day 6 indicated no differences among the groups concerning time spent in the target quadrant ($F_{(3,20)} = 1.58$; $p > 0.10$) or number of entries into the target quadrant ($F_{(3,20)} = 0.96$; $p > 0.10$).

Divalinal treatment

Figure 4*A* presents the mean latencies to find the pedestal for each group of the second experiment. On the first day of training, the groups did not differ; however, across the subsequent 5 d of acquisition, there were differences among the groups. Those animals that were continuously infused with aCSF, or nontreated controls, revealed steady improvement in performance, whereas members of the two groups treated with Divalinal performed very poorly and required significantly longer latencies to find the pedestal. The groups × days interaction was also significant and indicated that the groups treated with Divalinal were different from the groups infused with aCSF and the control group on days 4–6. *Post hoc* analyses indicated that the rate of improvement in acquisition performance was substantially greater for those animals that received aCSF and the noninfused controls compared with the Divalinal-treated groups. Figure 5 offers representative examples of performance by an animal from each of the four groups on day 6 of acquisition. Those rats treated with 5.0 or 0.5 nmol/hr Divalinal (Fig. 5*A*,*B*, respectively) evidenced much poorer search strategies than those animals infused with aCSF (Fig. 5*C*) or the noninfused controls (Fig. 5*D*). Several members of the 5.0 nmol/hr Divalinal group displayed a persistent tendency to swim near the walls of the maze (positive thigmotaxis) during training trials. This was seldom observed in the control animals or those infused with aCSF.

The statistical analyses to support these conclusions indicated a significant difference among the groups ($F_{(3,20)} = 3.08$; $p < 0.05$), a days effect over the initial 6 d of acquisition ($F_{(5,100)} = 37.16$; $p < 0.0001$), and a significant groups × days interaction ($F_{(15,100)} = 4.18$; $p < 0.0001$). Thus, by day 6, members of the two groups treated with Divalinal revealed significantly slower latencies to find the pedestal than those rats infused with aCSF and the noninfused control group ($F_{(3,20)} = 3.93$; $p < 0.05$). By day 13, 7 d

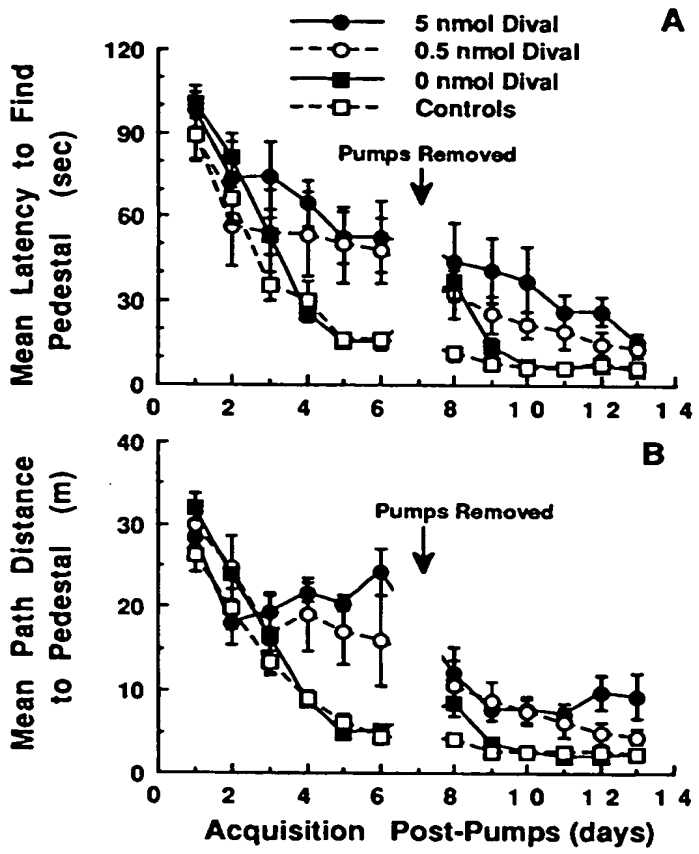

Figure 4. Mean ± SEM group changes in latency (*A*) and swim distance (*B*) to find the submerged pedestal in a circular water maze task during 6 d of acquisition training with osmotic pumps in place and 6 additional acquisition days with the pumps removed. All treatments were intracerebroventricularly delivered via osmotic pump at the indicated doses in a volume of 1 μl/hr aCSF. Those animals treated with 5.0 and 0.5 nmol/hr Divalinal (*Dival*) revealed significant deficits in performance compared with members of the control group (*0 nmol Dival*) and nontreated controls on days 4–6 ($p < 0.05$). After pump removal, the location of the submerged pedestal was shifted to the opposite quadrant for each animal. By day 13 of training, there were no differences in latencies to find the pedestal among the groups; however, those rats that had been treated with 5.0 nmol of Divalinal continued to reveal significantly longer swim distances to find the pedestal than members of the other three groups ($p < 0.05$). Each group consisted of six rats surgically prepared with 7 d osmotic pumps and were provided 1 d of recovery before initiation of acquisition training.

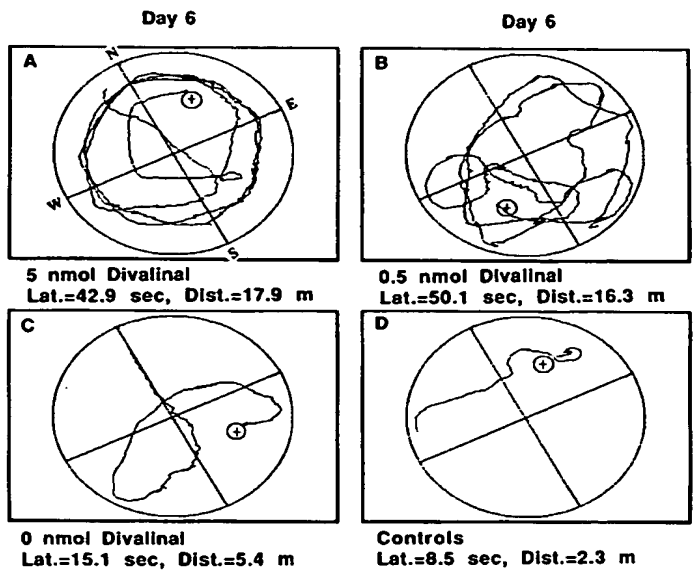

Figure 5. Representative examples of search patterns in the circular water maze by one member of the group treated with 5.0 (*A*) and one treated with 0.5 (*B*) nmol/hr Divalinal during day 6 of acquisition; also, one member from the control group (*C*) infused with aCSF (*0 nmol Divalinal*) and a member of the nontreated control group (*D*). All treatments were intracerebroventricularly delivered via osmotic pump in a volume of 1 μl/hr aCSF. Those animals treated with Divalinal performed poorly compared with members of the groups infused with aCSF or nontreated controls. Specifically, the search pattern strategies of the Divalinal-treated rats were not as sophisticated as the control animals and often included positive thigmotaxis (persistent swimming near the walls of the maze), as evidenced by the animal from the group treated with 5.0 nmol/hr Divalinal (*A*). Each group consisted of six rats prepared with 7 d osmotic pumps that infused at a rate of 1 μl/hr aCSF. Members of the fourth group served as nontreated controls.

after pump removal (Fig. 4*A*), the groups did not differ with respect to latencies to find the pedestal ($F_{(3,20)} = 1.66; p > 0.20$).

Figure 4*B* displays the mean ± SEM distance swam to find the submerged pedestal, and there were differences among the groups. On the first day of training, the groups did not differ; however, by the sixth day of acquisition training, those animals that received Divalinal performed poorly compared with those rats that received aCSF or the noninfused controls ($F_{(3,20)} = 7.22; p < 0.005$). There was improved performance over days ($F_{(5,15)} = 31.10; p < 0.0001$), and the groups × days interaction was also significant ($F_{(15,100)} = 6.29; p < 0.0001$) and indicated that the noninfused control group and those animals that received aCSF were superior to both Divalinal groups on days 4–6. Finally, the Divalinal groups did not differ from each other, nor did the aCSF and noninfused control groups differ. By day 13 of training (Fig. 4*B*), there remained differences among the groups concerning distance swam ($F_{(3,20)} = 3.03; p < 0.05$). Those rats treated with the 5.0 nmol/hr dose of Divalinal displayed significantly longer swim distances to find the pedestal than members of the other groups.

Results from the probe trials conducted at the conclusion of acquisition training on day 6 are presented in Figure 6. There were differences among groups concerning time spent within the target quadrant ($F_{(3,20)} = 3.25; p < 0.05$) (Figure 6*A*). *Post hoc* analyses indicated that those animals treated with the 5.0 and 0.5 nmol doses of Divalinal revealed significantly less time in the target quadrant (37.1 ± 2.6 and 35.2 ± 2.8 sec, respectively) than those infused with aCSF or the control group (45.8 ± 3.7 and 46.8 ± 2.1 sec, respectively). Other comparisons were not different. Figure 6*B* displays the number of entries into the target quadrant by members of each group. There were no differences among the groups on this measure.

Perforant path knife cuts

Those rats that received bilateral PP cuts and were subsequently treated with Norleucinal (PP/Norl) revealed significantly shorter latencies (Fig. 7*A*) and swim path distances (Fig. 7*B*) than rats prepared with bilateral PP cuts and infused with aCSF (PP/aCSF). In turn, members of the PP/Norl group revealed significantly slower acquisition curves than the group that received bilateral knife cuts to the neocortex, followed by infusion of aCSF (Cor/aCSF), but were equivalent with neocortex knife cut rats

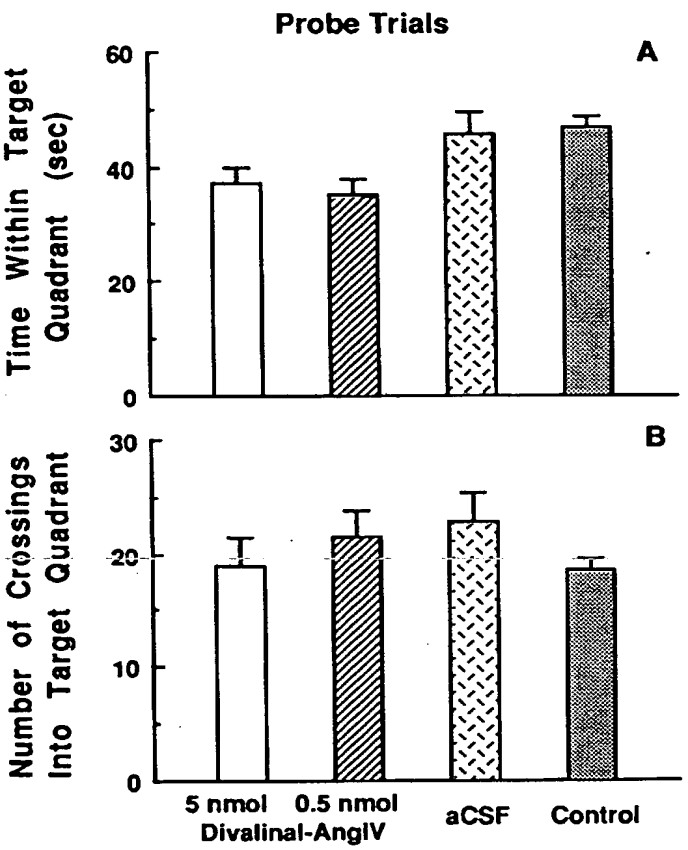

Figure 6. Mean ± SEM group changes and time spent within the correct (target) quadrant (*A*) and the number of entries into the quadrant (*B*) during one probe trial conducted at the conclusion of training trials on day 6 of acquisition for animals continuously intracerebroventricularly treated with 5.0, 0.5, or 0 nmol/hr Divalinal via osmotic pump for 6 d and nontreated controls. Those animals treated with 5.0 and 0.5 nmol/hr doses of Divalinal indicated significantly less time spent within the target quadrant compared with those rats that received intracerebroventricular infusion of aCSF or the nontreated control animals ($p < 0.05$). The groups did not differ with respect to number of entries into the target quadrant.

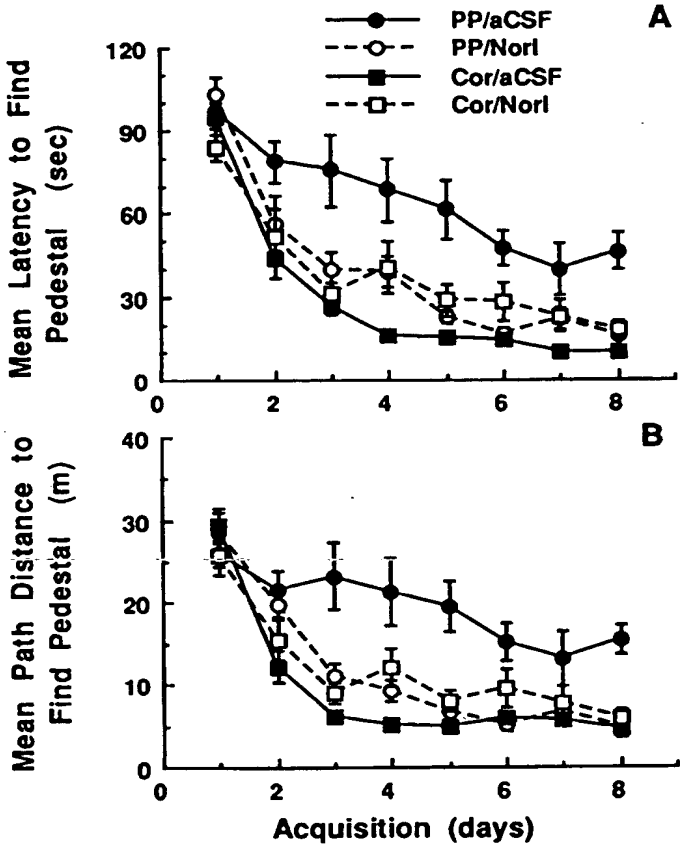

Figure 7. Mean ± SEM group changes in latencies (*A*) and swim distances (*B*) to find the submerged pedestal in the circular water maze tasks during 8 d of acquisition training by two groups of animals surgically prepared with bilateral PP knife cuts and intracerebroventricular guide cannulas and treated intracerebroventricularly with bolus injections, 2 μl of aCSF or 1.0 nmol of Norleucinal in 2 μl of aCSF, 5 min before the initiation of training trials on each day of acquisition. Two additional groups of animals served as controls and received bilateral knife cuts to the neocortex immediately superior to the PP and were also treated with aCSF or Norleucinal 5 min before training trials each day. Those animals prepared with PP knife cuts and treated with Norleucinal displayed an acquisition curve not different from the control groups prepared with neocortex knife cuts and treated with Norleucinal or aCSF. In contrast, those rats that received PP knife cuts and were infused with aCSF displayed significant impairment in acquisition of the spatial memory task with respect to latencies ($p < 0.001$) and swim distances ($p < 0.001$) to find the submerged pedestal. These differences became evident by day 3 of acquisition training and persisted during subsequent days. Each group consisted of eight rats.

that received Norleucinal (Cor/Norl). A 4(groups) × 8(acquisition days) ANOVA of these data revealed a groups effect ($F_{(3,28)} = 11.16$; $p < 0.001$), a days effect ($F_{(7,196)} = 74.7$; $p < 0.001$), and an interaction effect ($F_{(21,196)} = 2.42$; $p < 0.001$). *Post hoc* analyses indicated that rats in the PP/aCSF group displayed the longest latencies to find the pedestal compared with the other groups on days 2–8. The Cor/aCSF group revealed the shortest latencies on days 2–5 and 7. Members of the PP/Norl and Cor/Norl groups did not differ in their patterns of acquisition. By day 8, the PP/Norl, Cor/aCSF, and Cor/Norl groups did not differ (10.9 ± 1.6, 16.5 ± 3.8, and 18.5 ± 3.4 sec, respectively), whereas the PP/aCSF group indicated significantly longer latencies to find the submerged pedestal (46.8 ± 6.4 sec) ($F_{(3,28)} = 14.77$; $p < 0.001$).

Figure 8 provides representative examples of performance by an animal from each of these four groups on day 8 of acquisition. Those rats prepared with PP knife cuts and treated with aCSF (Fig. 8*A*) displayed much poorer search strategies than PP knife cut rats treated with Norleucinal (Fig. 8*B*) or neocortex knife cut rats treated with aCSF or Norleucinal (Fig. 8*C*,*D*, respectively).

Path distance analyses indicated similar group differences as reported above. There was a groups effect ($F_{(3,28)} = 8.33$; $p < 0.001$), a days effect ($F_{(7,196)} = 63.53$; $p < 0.001$), and an interaction effect ($F_{(21,196)} = 64.79$; $p < 0.001$). *Post hoc* analyses indicated that, by days 5–8 of acquisition, the Cor/aCSF, PP/Norl, and Cor/Norl groups did not differ. Members of all three of these groups displayed significantly shorter swim distances than members of the PP/aCSF group ($F_{(3,28)} = 10.08$; $p < 0.001$). By day 8, these mean values were 4.8 ± 0.8, 5.1 ± 1.2, 6.0 ± 0.9, and 14.3 ± 2.3 m for the Cor/aCSF, PP/Norl, Cor/Norl, and PP/aCSF groups, respectively. Probe trials conducted on day 8 indicated no differences among groups concerning time spent in the target quadrant ($F_{(3,28)} = 1.36$; $p > 0.10$) or the number of entries into the target quadrant ($F_{(3,28)} = 2.36$; $p > 0.05$).

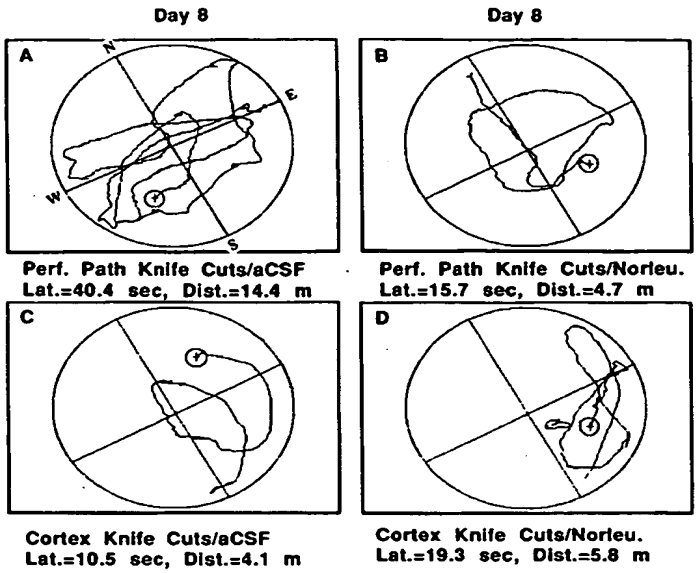

Figure 8. Representative examples of search patterns in the circular water maze by two members of the group prepared with bilateral PP knife cuts. One animal received intracerebroventricular administration of aCSF (*A*), and one member was treated with Norleucinal (*B*). For comparison purposes, two rats from the group prepared with bilateral neocortex knife cuts are also presented. One of these animals was from the group that was administered aCSF (*C*) and one that received Norleucinal (*D*). The animal that was prepared with PP knife cuts and was subsequently treated with Norleucinal displayed a search pattern that was equivalent with those by animals prepared with neocortex knife cuts and administered aCSF or Norleucinal. In contrast, those animals prepared with PP knife cuts and administered aCSF displayed significantly impaired search strategies ($p < 0.001$). Each group consisted of eight rats.

DISCUSSION

The hippocampus has been shown to play an important role in spatial learning and memory (Morris, 1981; McNaughton et al., 1986; Whishaw, 1987; Whishaw and Maaswinkel, 1998; Nadel 1991; Bures et al., 1997). Within the hippocampus, the CA_1 field (Sutherland et al., 1983; McNaughton et al., 1989; Morris et al., 1990; Jarrard, 1993) and, to a lesser extent the CA_3 field (Sutherland et al., 1983; Stubley-Weatherly et al., 1996), are involved in mediating these cortical functions. Related to these observations NMDA receptors are present in high densities on several hippocampal cell types, including CA_1–CA_3 pyramidal cells. The application of NMDA receptor antagonists has been shown to block the acquisition of spatial learning in rodents (Morris et al., 1986; Davis et al., 1992). The AT_4 receptor is heavily distributed within the hippocampus, as well as neocortex, cerebellum, and forebrain cholinergic structures. Of particular relevance to cognitive processing is the possibility that AT_4 receptors reside on both pyramidal and granular cells of the hippocampus and medial septal cholinergic neurons that innervate the neocortex and hippocampus. Support for this notion is offered by the observation that AT_4 agonists induce fos expression in these cells (Roberts et al., 1995), and iontophoretic application of AT_4 agonists drives these cells (Albrecht et al., 1997a,b). The linkage to cholinergic neurons is supported by the autoradiographic demonstration that AT_4 receptors colocalize with acetylcholinesterase in the spinal cord and the medial forebrain in monkeys (Møeller et al., 1996). Further, recent data from our laboratory indicate that SN56 cells, a cholinergic nucleus basalis–neuroblastoma hybrid (Hammond et al., 1996), possess particularly abundant AT_4 receptors (B_{max} = 1.74 ± 0.04 pmol/mg protein; K_d = 2.07 ± 0.14 nM; mean ± SEM; n = 3). This localization suggests that AT_4 receptors may modulate neurotransmission of glutaminergic and cholinergic synapses (glutamate is the neurotransmitter used by both hippocampal pyramidal and granule cells). Thus, activation of AT_4 receptors may produce changes in neurotransmitter release, postsynaptic receptors, receptor–intracellular signal coupling, or a combination thereof.

The present investigation initially determined that chronic intracerebroventricular delivery of Nle^1-AngIV facilitated acquisition of the circular water maze task of spatial memory during the initial 2 d of training. In contrast, chronic intracerebroventricular delivery of Divalinal significantly interfered with normal acquisition of this task during days 4–6 of training. This Divalinal-induced impairment was not evident during 6 additional days of training after pump removal, suggesting that this deficit in acquisition was reversible. These differences in acquisition could not be attributed to motor and/or sensory impairment as measured by performances on a motor test battery and visible platform protocols. Further, acute intracerebroventricular treatment with Norleucinal restored normal acquisition of a spatial memory task in animals prepared with bilateral perforant path knife cuts.

One potential mechanism underlying these AT_4 agonist effects relates to their ability to enhance cerebral blood flow. Increases in cerebral blood flow have been positively correlated with cognition. Angiotensin IV (Haberl et al., 1991; Kramár et al., 1997) and Norleucinal (Kramár et al., 1998) have been shown to increase cerebral blood flow by vasodilation of arterioles. These increments in blood flow appear to be nitric oxide-dependent. Thus, the presently noted improvement in memory could be mediated via elevations in cerebral blood flow. Along these lines, de la Torre (1994) has suggested that Alzheimer's disease may, in part, be caused by distortion of brain capillaries that prevent normal blood flow, thus producing ischemia with consequential damage to CA_1 field cells and other ischemic sensitive brain structures. Thus, increased blood flow to intact hippocampal neurons, as well as other neurons, may facilitate performance. A complimentary hypothesis by Sato and Sato (1995) proposes that cholinergic fibers possessing cell bodies within the NBM and the medial septum normally release acetylcholine within the hippocampus and neocortex, which in turn produces vasodilation and accompanying elevations in cerebral blood flow. Application of an AT_4 agonist could potentially stimulate cholinergic neurons in the NBM and the medial septum, where there are high densities of AT_4 receptors, reinstating normal acetylcholine release within the hippocampus and neocortex and, in turn, incrementing blood flow.

A second possible explanation relates to numerous studies that have demonstrated structural changes in the architecture of synaptic connections concomitant with the development of learning and memory (for review, see Agnihotri et al., 1998). These changes may be mediated by adhesive molecules that determine cell-to-cell and cell-to-extracellular matrix interactions in the brain. Recent data from our laboratory indicate that the AT_4 receptor plays a potentially pivotal role in the restructuring of the extracellular matrix in numerous tissues, including the brain (M. S. Cummings, J. M. Hanesworth, S. E. Hunter, and J. W. Harding, unpublished observations). Specifically, AT_4 receptors mediate the expression of matrix metalloproteinases (MMPs), their inhibitors, and members of the plasminogen-plasmin cascade, which are responsible for MMP activation. These observa-

tions raise the possibility that the effect of AT_4 activation may, in part, result from synaptic remodeling.

The majority of strategies concerned with the development of a pharmacological treatment for cognitive dysfunction have been stimulated by our current knowledge of long-term potentiation (LTP) processes. This LTP process by which synaptic strength is augmented in an activity-dependent manner has been correlated to cognitive processes (Lynch et al., 1983; Malenka et al., 1988; Izquierdo, 1993; Rison and Stanton, 1995; Wayner et al., 1995). The interplay of acetylcholine with LTP appears to be at the level of intracellular calcium and/or pyramidal cell depolarization. Working via muscarinic receptors, acetylcholine both increases intracellular calcium via IP_3-dependent mechanisms and depolarizes by attenuating potassium efflux, which acts to prime the cells for LTP. The importance of this process is illustrated by the effectiveness of muscarinic blockers as amnesiacs. Interestingly, AT_4 agonists can compensate for these muscarinic receptor antagonist-induced deficits (Pederson et al., 1998), suggesting that either acetylcholine release is augmented so that the effect of competitive muscarinic antagonists are overcome or, more likely, these agonists act independently to elevate intracellular calcium and "prime" the system to respond more effectively to glutamate. This notion is supported by the observation that AT_4 agonists increase intracellular calcium in cardiac myocytes, another excitable cell (B. K. Slinker, J. W. Harding, and S. Simasko, unpublished observations). Consistent with this idea are recent results indicating that AT_4 agonists facilitate LTP both *in vitro* (E. A. Kramár, unpublished observations) and *in vivo* (Ikeda et al., 1998). Thus, one pharmacological approach concerns enhancing cholinergic neurotransmission. Prototype drugs include acetylcholinesterase inhibitors, such as Cognex (Parke-Davis, Morris Plains, NJ; Warner-Lambert, Morris Plains, NJ), Aricept (Eisai, Tokyo, Japan; Pfizer, Groton, CT), Excelon (Novartis, Summit, NJ), Mentane (Hoechst Marion Roussel, Frankfurt am Main, Germany), and Metrifonate (Bayer, Wuppertal, Germany), which block acetylcholine removal from the synaptic cleft. A related strategy utilizes acetylcholine analogs possessing increased metabolic stability such as Memric (SmithKline Beecham, Upper Merion, PA), Xanomeline (Eli Lilly, Indianapolis, IN), and CI979 (Warner-Lambert) (for review, see Marx, 1996).

Our laboratory is presently developing nonpeptidic agonists that bind with high affinity to brain-specific AT_4 receptor subtypes. Results from the present investigation provide an initial demonstration of the potentially important role of the brain AT_4 receptor system in normal cognitive function and encourage the possible use of AT_4 receptor agonists to facilitate cognitive performance in compromised individuals.

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IN THE MATTER OF
US Serial No: 09/147,490
entitled"Neuroactive Peptide"

EXHIBIT 9

This is Exhibit 9 referred to in Clause 18 of the Statutory Declaration Siew Yeen Chai dated 13th Day of January 2004.

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Structure-Activity Study of LVV-Hemorphin-7: Angiotensin AT_4 Receptor Ligand and Inhibitor of Insulin-Regulated Aminopeptidase

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ABSTRACT

The decapeptide LVV-hemorphin-7 binds with high affinity to the angiotensin IV (Ang IV) receptor (AT_4 receptor), eliciting a number of physiological effects, including cellular proliferation and memory enhancement. We have recently shown that the AT_4 receptor is identical to insulin-regulated aminopeptidase (IRAP) and that both LVV-hemorphin-7 and Ang IV inhibit the catalytic activity of IRAP. In the current study, a series of alanine-substituted and N- or C-terminally modified analogs of LVV-hemorphin-7 were evaluated for their abilities to compete for ^{125}I-Ang IV binding in sheep adrenal and cerebellar membranes. Selected analogs were also analyzed for binding to recombinant human IRAP and inhibition of IRAP aminopeptidase activity. C-Terminal deletions of LVV-hemorphin-7 resulted in modest changes in affinity for IRAP, whereas deletion of the first three N-terminal residues abolished binding. Monosubstitutions of Tyr^4 and Trp^6 with alanine resulted in a 10-fold reduction in affinity. Competition binding studies using recombinant human IRAP demonstrated the same rank order of affinity as obtained for the ovine tissues. All LVV-hemorphin-7 analogs tested, except for Leu-Val-Val-Tyr, inhibit the cleavage of the synthetic substrate, leucine β-naphthylamide, by IRAP, with K_i values between 56 and 620 nM. We find that the Val^3 residue is crucial for LVV-hemorphin-7 binding to IRAP, whereas the C-terminal domain seems to play a minor role. The current study highlights the minimal residues necessary for binding and inhibition of IRAP and provides a basis to design peptidomimetic analogs for experimental and potentially clinical use.

A range of physiological functions are associated with Ang IV, including the facilitation of memory (Braszko et al., 1988; Wright et al., 1993, 1999), modulation of sodium uptake in the kidney (Hamilton et al., 2001), and vasodilatory effects (Haberl et al., 1991; Kramar et al., 1997, 1998). These actions are mediated by a specific binding site that has been termed the AT_4 receptor. We previously isolated an alternative AT_4 ligand, LVV-hemorphin-7 (LVVYPWTQRF), from the sheep cerebral cortex using a multistep procedure of reverse-phase and ion-exchange chromatography based on its ability to compete with ^{125}I-Ang IV for the AT_4 receptor (Moeller et al., 1997). LVV-hemorphin-7 shares identical sequence to residues 30 to 39 of sheep β-globin and residues 32 to 41 of the β-, δ-, γ-, and ε-human globin. Various studies have demonstrated that LVV-hemorphin-7 mimics many biological actions of Ang IV. At the cellular level, LVV-hemorphin-7 stimulates DNA synthesis in SK-N-MC cells (Mustafa et al., 2001), whereas in hippocampal slices, the decapeptide enhances the potassium-evoked release of acetylcholine (Lee et al., 2001). We have recently demonstrated that central administration of LVV-hemorphin-7 enhances spatial learning (J. Lee, A. L. Albiston, A. M. Allen, F. A. Mendelsohn, S. E. Ping, G. L. Barrett, M. Murphy, M. J. Morris, S. G. McDowall, and S. Y. Chai, manuscript submitted for publication).

We have identified the AT_4 receptor as the transmembrane enzyme insulin-regulated aminopeptidase (IRAP) via mass spectral analysis of tryptic peptides generated from AT_4 receptor purified from bovine adrenal membranes (Albiston et al., 2001). Analysis of the biochemical and pharmacological properties of IRAP confirm that it is the AT_4 receptor. We have also demonstrated that both Ang IV and LVV-hemorphin-7 inhibit the catalytic activity of IRAP, suggesting enzyme inhibition as one mechanism by which AT_4 ligands exert their effects (Albiston et al., 2001). For coherence, we describe the previously

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[1] J.L. and T.M. contributed equally to the work.
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ABBREVIATIONS: Ang IV, angiotensin IV; AT_4, angiotensin IV receptor; IRAP, insulin-regulated aminopeptidase; Leu-β-Na, Leu-β-naphthylamide; HEK, human embryonic kidney.

named AT_4 receptor agonists such as Ang IV and LVV-hemorphin-7 as AT_4 ligands and the AT_4 receptor as IRAP.

IRAP belongs to the M1 family of zinc metallopeptidases that is characterized by the zinc binding motif $HEXXH(X)_{18}$-E and the xopeptidase motif GXMEN. IRAP is a type II membrane-spanning protein such that when at the plasma membrane the catalytic site is extracellular (Keller et al., 1995). The enzyme was initially defined as specifically cleaving the N-terminal amino acid CysXaa-, in which the half-cystine residue is involved in a disulfide loop, notably in oxytocin or vasopressin, but in vitro has also been demonstrated to cleave a range of peptides not containing disulfide loops (Matsumoto and Mori, 1998; Matsumoto et al., 2000). Our preliminary studies indicate that AT_4 ligands are not cleaved by IRAP (R. A. Lew, T. Mustafa, S. Ye, S. G. McDowall, S. Y. Chai, and A. L. Albiston, manuscript submitted for publication).

Considering the wide-ranging effects mediated by AT_4 ligands, an understanding of the structural requirements for the ligand-enzyme interaction will be beneficial for the design of metabolically stable inhibitors of IRAP. The critical amino acids required for Ang IV binding to IRAP have been identified (Sardinia et al., 1993, 1994; Krishnan et al., 1999). The presence of an amino-terminal valine, and more precisely, a primary α-amine in the L-amino acid conformation in position 1, seems to be important in the binding process. Glycine substitutions at positions 1, 2, or 3 of Ang IV greatly reduce affinity for IRAP, whereas substitutions at positions 4, 5, or 6 of Ang IV have little effect (Sardinia et al., 1993). Moreover, N-terminal elongation of Ang IV results in a marked reduction in affinity, whereas C-terminally extended peptides bind to the receptor with an affinity similar to that of the native ligand (Sardinia et al., 1993). Thus, the N-terminal residues of the Ang IV peptide are critical for receptor binding, whereas the C-terminal portion plays a less critical role.

Interestingly, despite a similar binding affinity for IRAP, LVV-hemorphin-7 (LVVYPWTQRF) shares little sequence homology to Ang IV (VYIHPF). Considering that this biologically active peptide is more stable than Ang IV (Moeller et al., 1999), LVV-hemorphin-7 may be a useful template for the design of peptidomimetics targeting the IRAP protein. In the current study, we set out to determine the structural requirements of LVV-hemorphin-7 binding to IRAP. To achieve this aim, a series of N-and C-terminally modified and alanine-substituted analogs of LVV-hemorphin-7 were screened for their abilities to compete for ^{125}I-Ang IV binding in sheep adrenal and cerebellar membranes. Moreover, selected truncated LVV-hemorphin-7 analogs were also analyzed for their ability to bind to and inhibit the recombinant form of human IRAP.

Materials and Methods

Synthesis and Preparation of Peptides

Truncated analogs of LVV-hemorphin-7 and Val-Tyr-Pro-motif extended peptides were synthesized by Mimotopes (Clayton, Victoria, Australia). The N-terminally extended analogs and Ala-substituted analogs of LVV-hemorphin-7 were synthesized in the peptide laboratory at the Howard Florey Institute (University of Melbourne, Parkville, Victoria, Australia), using the continuous flow Fmoc-methodology (Wade et al., 2001). Peptides were dissolved in 0.05 M acetic acid and stored as 1 mM stock solutions at −20°C. The fluorescent substrate Leu-β-naphthylamide (Leu-β-NA), its cleavage product β-naphthylamine, and other reagents were purchased from Sigma Chemical (Castle Hill, NSW, Australia).

Tissu Samples

Sheep adrenal glands and cerebellum obtained from the abattoir were frozen in isopentane on dry ice at −40°C and stored at −80°C.

Expression of Human IRAP in Human Embryonic Kidney (HEK) 293T Cells

HEK293T cells were transiently transfected with either pCI-IRAP (a gift from M. Tsujimoto, Department of Obstetrics and Gynaecology, Nagoya University School of Medicine, Japan) or empty vector using LipofectAMINE transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Membrane Preparation. Membranes were prepared as described previously (Mustafa et al., 2001). In brief, tissues and transfected cells were suspended in 50 mM Tris-HCl pH 7.4, homogenized for 10 s, and centrifuged at 600*g* for 5 min at 4°C to remove cellular debris. The supernatant was incubated for 20 min at 65°C followed by centrifugation at 50,000*g* for 20 min at 20°C. Membranes were resuspended in 50 mM Tris, 5 mM EDTA, 150 mM sodium chloride buffer containing 100 μM phenylmethylsulfonyl fluoride, 20 μM bestatin, 100 μM phenanthrolene, and 0.1% bovine serum albumin.

Western Blot Analysis of IRAP in Ovine Adrenal and Cerebellum Membranes

Ovine adrenal and cerebellum crude membranes (200 μg of total protein) were run on SDS-polyacrylamide gel electrophoresis. The resolved proteins were transferred to a Protran BA nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) and immunodetected using an in-house rabbit anti-IRAP polyclonal antibody (raised against amino acids 25–47 of human IRAP). The primary antibody was detected using horseradish peroxidase-conjugated sheep anti-rabbit secondary antibody (Chemicon International, Temecula, CA); enhanced chemiluminescence was used to detect conjugated horseradish peroxidase activity and was captured using a luminescent image analyzer LAS-1000 plus (FujiFilm, Kanagawa, Japan).

Binding Assays

Competition. Crude membranes (20 μg for transfected cells and 65 μg for ovine tissues) of protein were incubated with 0.5 μCi/ml of ^{125}I-Ang IV and increasing concentrations (10^{-12}–10^{-5} M) of unlabeled peptide, for 2 h at 37°C. Bound and free radioligand was separated using the standard filtration method as described previously (Moeller et al., 1997). The radioligand binding data were analyzed using the GraphPad Prism program (GraphPad Software Inc., San Diego, CA) to determine the IC_{50} value for each analog. LVV-hemorphin-7 or Ang IV was included for each set of experiments serving as controls.

Saturation. Binding studies were carried out by incubating transfected cell membranes (2 μg) in the presence of increasing concentrations (1–12,000 pM) of ^{125}I-Ang IV, and nonspecific binding was determined in the presence of 10 μM of unlabeled Ang IV; K_d and B_{max} values obtained by Scatchard analysis. K_i values were obtained using the equation $IC_{50} = K_i(1 + [S]/K_d)$.

Enzyme Inhibition Assay

For enzyme activity assays, cell membranes were prepared as described above, omitting EDTA in the harvesting buffer. The membrane pellet was resuspended in 20 mM HEPES, 255 mM sucrose, 100 mM NaCl, pH 7.4, with protease inhibitors (10 μg/ml aprotinin, 10 μM leupeptin, 1 μM pepstatin, and 1 mM phenylmethylsulfonyl fluoride), snap frozen on dry ice, and stored at −70°C for up to 3 months.

Aliquots of crude membranes were thawed, centrifuged at 9000*g*

in a tabletop Microfuge at 4°C for 15 min, and the supernatant discarded. Membranes were resuspended in Tris-buffered saline (25 mM Tris-HCl, 125 mM NaCl, pH 7.4) containing 1% Triton X-100 at a protein concentration of 1 mg/ml, and rotated gently for at least 5 h at 4°C to solubilize membrane proteins. After solubilization, the membranes were pelleted by centrifugation as described above, the supernatant stored at 4°C, and used in assays within 24 h.

IRAP activity was monitored by the increase in fluorescence after cleavage of Leu-β-NA. Assays were performed in black 96-well microtiter plates: each well contained 2 μg of human IRAP-HEK293T-solubilized membrane protein, 25 μM Leu-β-NA, and the peptide of interest in a final volume of 200 μl of Tris-buffered saline. Reactions proceeded at 37°C for 30 min in a thermostated *f*Max fluorescence microplate reader (Molecular Devices Corp., Sunnyvale, CA), before reading the fluorescence (λexcitation = 320 nm, λemission = 420 nm). The ability of each peptide to inhibit IRAP was determined over a range of peptide concentrations (0.01–10 μM), with each concentration being assayed in triplicate in two separate experiments. Inhibitor constants (K_i) for competitive inhibitors were calculated from the relationship $IC_{50} = K_i(1 + [S]/K_m)$, where K_m for Leu-β-NA was previously determined from kinetic experiments to be 32.3 μM (R. A. Lew, T. Mustafa, S. Ye, S. G. McDowall, S. Y. Chai, and A. L. Albiston, manuscript submitted for publication).

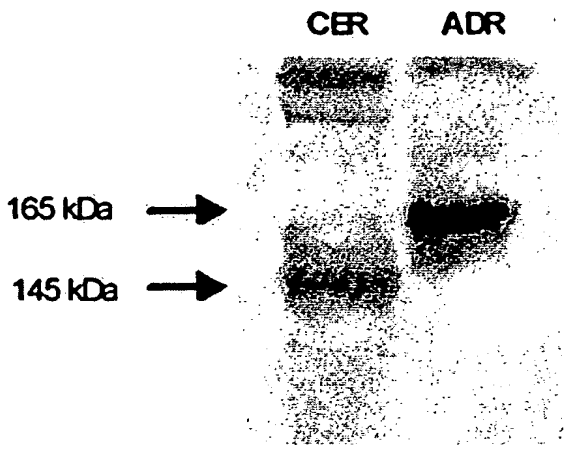

Fig. 1. Western blot analysis of IRAP in crude membrane preparations of ovine cerebral (cer) and adrenal (adr) tissues. Crude membrane preparations (200 μg), prepared as described under *Materials and Methods*, were submitted to SDS-polyacrylamide gel electrophoresis, blotted on to nylon membrane, and immunodetected with the anti-IRAP antibody and developed with enhanced chemiluminescence.

Statistics

The IC_{50} value for each peptide was determined and expressed as the mean ± S.E.M. (GraphPad Prism; GraphPad Software Inc.). Statistical differences between IC_{50} values for the various peptides were determined by one-way analysis of variance (GraphPad Prism). Where there was a significant effect between LVV-hemorphin-7 and the modified peptide ($P < 0.05$) on analysis of variance, Bonferroni's post hoc test was used to determine the significance of difference between the two peptides.

TABLE 1

Binding affinities (IC_{50}) of truncated analogs of LVV-hemorphin-7 for IRAP

Competition binding studies on [^{125}I]-Ang IV binding to sheep adrenal or cerebellar membranes in the presence of increasing concentrations of N-terminal or C-terminal truncated peptides were performed as described under *Materials and Methods*. Values are the mean ± S.E.M.of three experiments performed in duplicate.

Peptides	Sheep Adrenal IC_{50}	Sheep Cerebellum IC_{50}
	nM	
Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe (LVV-hemorphin-7)	17.6 ± 6.2	5.0 ± 0.7
C-Terminal deleted peptides		
Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg	6.8 ± 1.0	10.7 ± 1.1
Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln	34.0 ± 5.7	23.2 ± 2.1
Leu-Val-Val-Tyr-Pro-Trp-Thr	8.2 ± 2.3	3.9 ± 1.9
Leu-Val-Val-Tyr-Pro-Trp	13.3 ± 0.58	5.9 ± 1.9
Leu-Val-Val-Tyr-Pro	46.1 ± 13.8	46.0 ± 7.1**
Leu-Val-Val-Tyr	185.9 ± 82.3***	189.6 ± 60.4***
N-Terminal deleted peptides		
Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	14.7 ± 3.2	35 ± 6.1
Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	0.98 ± 0.3*	0.50 ± 0.1*
Tyr-Pro-Trp-Thr-Gln-Arg-Phe	N.D. (>μM)	N.D. (>μM)

N.D., not detectable.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$: significantly different from LVV-hemorphin-7

TABLE 2

Binding affinities (IC_{50}) of Val-Tyr-Pro-extended analogs for IRAP

Competition binding studies on [^{125}I]-Ang IV binding to sheep adrenal or cerebellar membranes in the presence of increasing concentrations of Val-Tyr-Pro-extended analogs were performed as described under *Materials and Methods* ($n = 3$ for each peptide).

Peptides	Sheep Adrenal IC_{50}	Sheep Cerebellum IC_{50}
	nM	
Val-Tyr-Pro-extended peptides		
Val-Tyr-Pro	N.D. (>μM)	N.D. (>μM)
Val-Tyr-Pro-Trp	17.2 ± 4.5*	19.0 ± 3.6*
Val-Tyr-Pro-Trp-Thr	1.9 ± 0.9	1.1 ± 0.1
Val-Tyr-Pro-Trp-Thr-Gln	1.2 ± 0.2	1.0 ± 0.1
Val-Tyr-Pro-Trp-Thr-Gln-Arg	4.1 ± 0.5	2.0 ± 0.7
Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	0.98 ± 0.3	0.5 ± 0.1
Leu-Val-Val-Tyr-Pro	46.1 ± 13.8*	46.1 ± 7.1*
Val-Tyr-Ile-His-Pro-Phe (Ang IV)	2.9 ± 0.8	1.9 ± 0.6

N.D., not detectable.
* Significantly different from Ang IV ($P < 0.05$).

TABLE 3

Binding affinities (IC_{50}) of alanine-substituted LVV-hemorphin-7 analogs for IRAP

Competition binding studies on [^{125}I]-Ang IV binding to sheep adrenal or cerebellar membranes in the presence of increasing concentrations of alanine-substituted analogs were performed as described under *Materials and Methods* (n = 3 for each peptide).

Peptides	Sheep Adrenal IC_{50}	Sheep Cerebellum IC_{50}
	nM	
Alanine-substituted peptides		
Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	26 ± 7.2	18.6 ± 6.5
Leu-Val-Val-Ala-Pro-Trp-Thr-Gln-Arg-Phe	236.4 ± 22.4*	221.7 ± 61.5*
Leu-Val-Val-Tyr-Ala-Trp-Thr-Gln-Arg-Phe	55.7 ± 15.1	57 ± 21.3
Leu-Val-Val-Tyr-Pro-Ala-Thr-Gln-Arg-Phe	273.9 ± 61.1*	140 ± 12.3*
Leu-Val-Val-Tyr-Pro-Trp-Ala-Gln-Arg-Phe	19.9 ± 7.7	9.4 ± 3.2

* Significantly different from LVV-hemorphin-7 ($P < 0.05$).

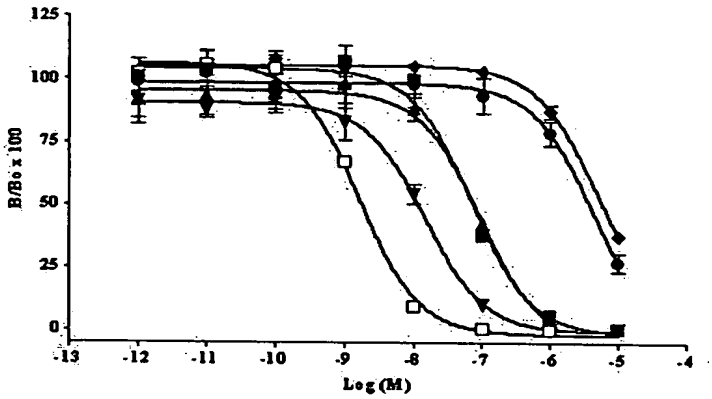

Fig. 2. Competition binding of ^{125}I-Ang IV binding to HEK293Tcells transfected with pCI-IRAP. Crude membranes were prepared, as described under *Materials and Methods*, and inhibition of ^{125}I-Ang IV binding to IRAP by peptides LVV-H7 (■), LVVYPWT (▲), VYPWTQRF (□), VYPWT (▼), LVVY (◆), and VYP (●) was performed. Values are the mean ± S.E.M. of three experiments performed in duplicate. B/Bo × 100 = percentage of available binding sites occupied.

Results

Affinity for Central and Peripheral IRAP Site. Western blot analysis clearly demonstrates a size difference between the sheep cerebellum and adrenal gland IRAPs as has previously been described for both the rat and bovine central and peripheral tissues (Keller et al., 1995; Zhang et al., 1999). The molecular mass of ovine adrenal IRAP is 165 kDa and ovine cerebellum IRAP is 145 kDa (Fig. 1). However, there were no statistically significant differences in the IC_{50} values obtained, between the cerebellum and adrenal IRAP, for any of the peptides tested (Tables 1-4).

C-Terminal Deletions of LVV-Hemorphin-7. Deletions of the C-terminal residues Phe^{10}, Arg^9, Gln^8, and Thr^7 (Leu^1-Val^2-Val^3-Tyr^4-Pro^5-Trp^6) from the full-length LVV-hemorphin-7 peptide did not significantly affect their affinities for IRAP, except for a modest decrease in affinity with deletion of the Arg^9 residue in cerebellar membranes. Subsequent removal of the Trp^6 and Pro^5 (Leu^1-Val^2-Val^3-Tyr^4) resulted in rightward shifts in the competition binding curves for both adrenal and cerebellar IRAP (5–10-fold for Leu^1-Val^2-Val^3-Tyr^4-Pro^5 and 23–50-fold for Leu^1-Val^2-Val^3-Tyr^4, relative to LVV-hemorphin-7) ($P < 0.01$) (Table 1).

N-Terminal Deletions of LVV-Hemorphin-7. Deletion of the Leu^1 residue of LVV-hemorphin-7 (Val^2-Val^3-Tyr^4-Pro^5-Trp^6-Thr^7-Gln^8-Arg^9-Phe^{10}) did not significantly affect its affinity for IRAP (Table 1). Serial deletion of the Val^2 residue (Val^3-Tyr^4-Pro^5-Trp^6-Thr^7-Gln^8-Arg^9-Phe^{10}) resulted in a 10-fold increase in the affinity for IRAP with respect to LVV-hemorphin-7 ($P < 0.05$). Subsequent removal of the Val^3 residue resulted in abolition of binding to IRAP (Table 1).

Val-Tyr-Pro-Motif Extended Peptides. Previous structure-activity studies with Ang IV revealed that the minimum requirement of binding to IRAP is the tripeptide VYI, which binds to IRAP with weak affinity (IC_{50} = 0.48 μM) (Sardinia et al., 1993). We therefore investigated the effect of sequential C-terminal or N-terminal extension of the tripeptide Val-Tyr-Pro, using amino acid sequences from LVV-hemorphin-7. In both the sheep adrenal and cerebellar membranes, Val-Tyr-Pro failed to compete for ^{125}I-Ang IV binding even at concentrations of >10 μM. The addition of Trp to the C terminus of the peptide (Val-Tyr-Pro-Trp) increased the affinity in both adrenal and cerebellar membranes (IC_{50} = 17.2 and 19.0 nM, respectively) (Table 2). Subsequent addition of a Thr residue (Val-Tyr-Pro-Trp-Thr) increased the affinity by a further 10-fold (IC_{50} = 1.9 and 1.1 nM, respectively), with no further increase with addition of the last three residues, Gln, Arg, and Phe. Addition of both Leu and Val to the N terminus of Val-Tyr-Pro (Leu-Val-Val-Tyr-Pro) increased the

TABLE 4

K_i values obtained for enzyme inhibition and competition binding of LVV-hemorphin-7 analogs with recombinant human IRAP

Competition binding studies on [^{125}I]-Ang IV binding to crude membranes in the presence of LVV-H7 analogs was performed as described under *Materials and Methods*. IRAP enzymatic activity was determined by the hydrolysis of the synthetic substrate, Leu-β-NA in the presence or absence of LVV-H7 analogs (n = 3 for each peptide).

Peptide	Enzyme Inhibition K_i	Competition Binding K_i
	nM	
Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	56	1.0
Val-Tyr-Pro-Trp-Thr	112	11
LVV-hemorphin-7	196	55
Leu-Val-Val-Tyr-Pro-Trp-Thr	560	73
Val-Tyr-Pro	620	3100
Leu-Val-Val-Tyr	N.D.	3400

N.D., not detected.

affinity to 46 nM in both adrenal and cerebellar membranes, respectively (Table 2).

Alanine-Substitution of LVV-Hemorphin-7. To determine the importance of specific residues at defined positions, residues 4 to 7 from LVV-hemorphin-7 were monosubstituted with alanine. Substitution of the Tyr^4 and Trp^6 residues with Ala resulted in a 10-fold decrease in affinity with respect to the parent peptide ($P < 0.05$) (Table 3). However, the replacement of the Pro^5 and Thr^7 residues had little effect on the peptide's affinity for the IRAP.

Analysis of LVV-Hemorphin-7 Analogs with Recombinant Human IRAP. Saturation binding studies using ^{125}I-Ang IV indicate that the IRAP-transfected cells contain a high-affinity Ang IV binding site with K_d = 1.8 nM and B_{max} = 5000 fmol/mg. Competition binding studies using recombinant human IRAP and selected LVV-hemorphin-7 analogs demonstrated the same rank order of affinity as obtained for the ovine tissues (Fig. 2). IRAP K_i values for the peptides were calculated (Table 4) and ranged between 1 and 3500 nM. The K_i (human IRAP) and IC_{50} (sheep IRAP) values for Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe are nearly the same (1.0 nM), whereas for the other truncated LVV-hemorphin-7 peptides the K_i (human IRAP) values obtained are higher than the IC_{50} (sheep IRAP) values (Tables 1 and 2). Therefore, although the rank order of the affinity of the selected LVV-hemorphin-7 analogs is the same for human IRAP and ovine IRAP, the relative affinities differ. This may reflect species-specific differences in the affinity of the different peptides for IRAP. All of the LVV-hemorphin-7 analogs tested, except for Leu-Val-Val-Tyr, inhibit the cleavage of the synthetic substrate Leu-β-NA, by IRAP, with K_i values between 56 and 620 nM (Fig. 3; Table 4).

Discussion

The current study aims to delineate the structural requirements for LVV-hemorphin-7 binding to IRAP and thus to extend the knowledge on the ligand-enzyme interaction in this system. This was achieved by initially screening a series of N- and C-terminally modified analogs of the decapeptide for their ability to competitively inhibit the binding of ^{125}I-Ang IV to sheep adrenal and cerebellar membranes. IRAP in the central nervous system is approximately 10% smaller compared with IRAP from peripheral tissues (Keller et al., 1995; Zhang et al., 1998). It has been suggested that in part this difference in size may be due to differential glycosylation. In this study, we demonstrated that in ovine tissues the same variation occurs, adrenal IRAP is 165 kDa and cerebellum IRAP is 145 kDa. The results from this study did not identify selectivity for either central or peripheral IRAP binding sites.

The results from the N-terminal deletion studies indicate that the Val^3 residue of LVV-hemorphin-7 is crucial for interaction with IRAP, because deletion of this residue completely abolishes binding to IRAP. Along these lines, Garreau et al. (1998) investigated the ability of LVV-hemorphin-7 and related peptides to inhibit ^{125}I-Ang IV binding in collecting duct principal cell membranes. They reported that the most potent competitors were LVV-hemorphin-7 and VV-hemorphin-7 (1.3 nM), whereas hemorphin-7 (YPWTQRF) failed to compete for ^{125}I-Ang IV binding sites. Similarly, deletion or substitution of the Val^1 residue from Ang IV significantly reduced its affinity for IRAP (Sardinia et al., 1993). Sardinia et al. (1994) suggested that the hydrophobic nature of the Val residue is necessary for Ang IV to bind to IRAP with high affinity. Indeed, a hydrophobic amino acid, Ile, in position 1 increased affinity, whereas the charged amino acid Glu decreased affinity for IRAP (Sardinia et al., 1994).

Because IRAP is an aminopeptidase, it is less surprising that the C-terminal residues do not seem to play an important role in the determination of the ligands affinity for IRAP. Deletion of the last four C-terminal residues of LVV-hemorphin-7 ($T^7Q^8R^9F^{10}$) do not significantly affect the pep-

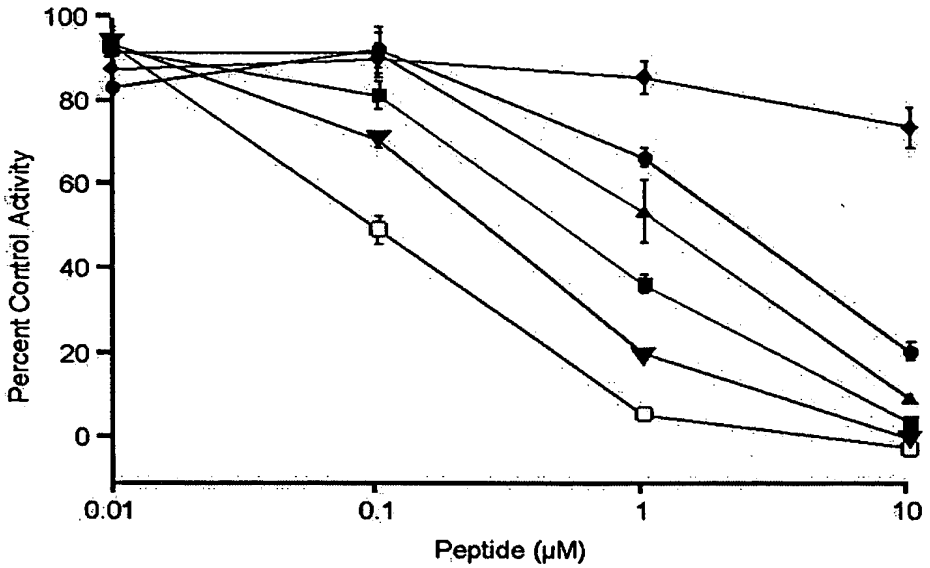

Fig. 3. Inhibition of IRAP catalytic activity by LVV-H7 analogs. Crude membranes were prepared from HEK293T cells transfected with pCI-IRAP. IRAP enzymatic activity was determined by the hydrolysis of the synthetic substrate Leu-β-NA. The substrate was added to solubilized membrane protein (2 μg) in the presence or absence of LVV-H7 analogs (symbols as for Fig. 2A) at the indicated concentrations and the fluorescence monitored for 30 min. Values are the mean ± S.E.M. of three experiments performed in duplicate.

tide's affinity for IRAP. In line with this, substitutions, deletions, or extensions of the C-terminal residues of Ang IV had little to modest effects on IRAP binding (Sardinia et al., 1993). These results support the notion that the N-terminal residues primarily determine the affinity of a ligand for IRAP.

To determine the influence of amino acid side chains on the ligand-enzyme interaction, a selected group of residues ($Tyr^4Pro^5Trp^6Thr^7$) from LVV-hemorphin-7 were substituted with alanine. Substitution of either Tyr^4 or Trp^6 with alanine results in a significant decrease in affinity, suggesting that these aromatic amino acids play a role in determining affinity for IRAP. The Tyr^2 residue in Ang IV is important for IRAP binding, due to its hydrophobic nature and planar geometry (Krishnan et al., 1999).

Sardinia et al. (1993) demonstrated that the tripeptide VYI is the minimum requirement for Ang IV binding to IRAP. Ang IV and LVV-hemorphin-7 sequence both share the sequence VY in the N terminus. This led us to investigate the binding of the tripeptide VYP, derived from the LVVYPWTQRF sequence, to IRAP. The VYP peptide displayed poor affinity for IRAP in both cerebellar and adrenal membranes. However, the addition of the hydrophobic amino acid Trp to the C terminus of VYP (VYPW) improves the affinity significantly. Taken together, the presence of a hydrophobic amino acid at the C-terminal end of VYP may be important for high-affinity binding. In support of this, a hydrophobic amino acid is required at position three of Ang IV to achieve high-affinity binding (Krishnan et al., 1999). Alternatively, the addition of the Leu^1Val^2 residues to the N terminus of VYP peptide also improves binding significantly. Thus, in the absence of the C-terminal residues, the Leu^1Val^2 amino acids may be important for binding to IRAP, possibly by altering the tertiary conformation of the VYP peptide to maximize peptide-enzyme interaction.

A limitation of competition binding studies to delineate the structural requirements for high-affinity binding to IRAP (AT_4 receptor) is that they are performed in the presence of chelating agents (phenanthroline and/or EDTA), whereas in vivo IRAP, a zinc metalloproteinase, is present with a bound zinc. Therefore, the enzyme inhibition assay is a useful system to assess the structural requirements for high-affinity binding of AT_4 ligands to IRAP in a biologically relevant context. AT_4 ligands, including LVV-hemorphin-7, are not substrates of IRAP, because we have recently demonstrated that HEK293T cells transfected with IRAP do not degrade these peptides beyond the level observed for mock-transfected cells (<15% over 4 h) (R. A. Lew, T. Mustafa, S. Y. Chai, and A. L. Albiston, unpublished data). LVV-hemorphin-7 and the five selected truncated analogs inhibited the aminopeptidase activity of IRAP in the same rank order as obtained for competition binding. However, the K_i values obtained for the truncated LVV-hemorphin-7 analogs using the enzyme inhibition and the competition binding assays differ markedly. The K_i values obtained from the enzyme inhibition assay were up to 100-fold lower compared with values obtained from the competition binding assay. Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe the most potent peptide in both the enzyme inhibition and the competition binding assays had K_i values of 56 and 1 nM determined from the respective assays. The differences in the K_i values obtained from the two assays is likely to be due to the presence or absence of zinc bound to IRAP altering the affinity of the LVV-hemorphin-7 analogs.

Beyond the prerequisite for a free N terminus, the requirements for substrate binding to IRAP are difficult to define. The enzyme has previously been defined as specifically cleaving the N-terminal amino acid CysXaa-, in which the half-cystine residue is involved in a disulfide loop, notably in oxytocin, vasopressin, and somatostatin (Herbst et al., 1997). N-Terminal cysteine residues seem to be the preferential targets for the enzyme; however, other peptides that possess N-terminal cysteine residues and intramolecular disulfide bonds, such as calcitonin and endothelin, are not cleaved by the enzyme. Other peptides that are readily cleaved by IRAP include Lys-bradykinin, met-enkephalin, dynorphin A, neurokinin A, and neuromedin B (Herbst et al., 1997), which possess a range of N-terminal residues. In contrast to AT_4 ligands the affinities of such substrates are in the mid-micromolar range, as is common with peptidases.

In conclusion, we have demonstrated that the Val^3 residue is crucial for LVV-hemorphin-7 binding to IRAP. This observation is in keeping with the suggestion that a hydrophobic amino acid is required at the N terminus for high-affinity binding to IRAP. In contrast, the C-terminal domain of LVV-hemorphin-7 does not seem to play an important role in determining ligands affinity for IRAP. The results from the current study indicate that the minimal sequence required for high-affinity binding and inhibition of IRAP is VYPWT. Modification of this truncated analog of LVV-hemorphin-7, using systematic cyclization and bicyclization, may yield specific, potent inhibitor(s) of IRAP.

Acknowledgments

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IN THE MATTER OF
US Serial No: 09/147,490
entitled"Neuroactive Peptide"

EXHIBIT 10

This is Exhibit 10 referred to in Clause 20 of the Statutory Declaration Siew Yeen Chai dated 13th Day of January 2004.

Before me: _____

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meaning of the Patents Act 1990.

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Protein-Tyrosine Phosphatase Inhibition by a Peptide Containing the Phosphotyrosyl Mimetic, *L-O*-Malonyltyrosine

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Summary: Peptides containing phosphonate based non-hydrolyzable phosphotyrosyl (pTyr) mimetics previously have been shown to be competitive inhibitors of protein-tyrosine phosphatases (PTPs). These agents suffer from low cellular penetration which is partially attributable to ionization of the phosphonate group at physiological pH. We have developed the non-phosphorus containing pTyr mimetic, *L-O*-malonyltyrosine (*L*-OMT) and herein demonstrate using a PTP 1B enzyme assay that it is superior to phosphonomethyl phenylalanine (Pmp) as a pTyr mimetic when incorporated into the hexamer peptide Ac-D-A-D-E-X-L-amide (X = D,L-Pmp, IC_{50} = 200 μM; X = *L*-OMT, IC_{50} = 10 μM). Prodrug protection of *L*-OMT as its carboxylic acid diester could potentially increase cellular penetration, thereby making this a valuable reagent for cellular studies.

The role of cell signalling in pathogenic processes is currently an active area of research, with kinase-dependent pathways being of particular importance. Protein-tyrosine phosphatases (PTPs) participate with protein-tyrosine kinases (PTKs) in the modulation of phosphotyrosyl (pTyr)-dependent signalling. The action of PTPs can be either inhibitory or stimulatory (2, 3), and mounting evidence suggests that some PTPs may enhance mitogenic signalling (4-8) and play roles in cellular transformation (9-13) and diabetes (14). Inhibitors of PTPs therefore represent attractive developmental targets both for their potential therapeutic value and their use as pharmacological tools.

We have approached the design of new PTP inhibitors by modifying peptide substrates so as to render them incapable of undergoing chemical transformation by the PTP. Replacement of the pTyr residue (1) in PTP-substrate peptides, with the non-hydrolyzable pTyr mimetic, phosphonomethyl phenylalanine (Pmp 2) (15), results in peptides which are competitive PTP inhibitors (16, 17). Pmp differs from pTyr in having a methylene substituted for the tyrosyl 4'-ester oxygen. We recently reported that substitution of the Pmp residue in one such hexameric inhibitor peptide with a difluoromethylene-containing analogue (difluorophosphonomethyl

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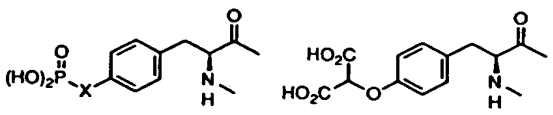
phenylalanine, F_2Pmp 3 (18-20)) resulted in a 1000-fold increase in inhibitory potency (IC_{50} = 100 nM against PTP 1B (21)). Because the difluorophosphonate moiety is di-ionized at physiological pH (22), transport across cell membranes is compromised and cellular studies of F_2Pmp-containing peptides have resorted to membrane permeablization (23) or microinjection (8) techniques. While prodrug protecting groups have been developed for phosphates (24-27) and phosphonates (28, 29), these protecting groups have not yet been extended to F_2Pmp-containing peptides, where synthetic challenges exist (Figure 1).

For the preparation of cell-permeable inhibitors, we therefore sought to devise an alternative pTyr mimetic which would be more amenable to facile prodrug derivatization. Relying on the extensive work of Sikorski et al. that utilized a malonyl group to mimic phosphate functionality in EPSP (5-enolpyruvoyl-shikimate-3-phosphate) synthase inhibitors (30), we designed the pTyr analogue, *L-O*-malonyltyrosine (*L*-OMT 4), and demonstrated its utility in the preparation of src homology 2 (SH2) domain inhibitory peptides (31). The malonyl structure contains two carboxylic acids instead of the phosphate group and as such, it can be readily protected as the di-ester for delivery across cell membranes. Once inside the cell esterase-mediated cleavage of the esters would liberate the active di-acid form. In the present study we synthesize and examine the PTP 1B inhibitory potency of the hexameric *L*-OMT-containing peptide, Ac-D-A-D-E-[*L*-OMT]-L-amide whose sequence is based on the structure of a high affinity PTP 1 substrate (17).

Materials and Methods

Materials. Protein tyrosine phosphatase 1B (PTP-1B) was obtained from Upstate Biotechnology Inc., (Lake placid, NY) and wheat germ agglutinin (WGA)-coupled to agarose was purchased from Vector Laboratories, Inc. (Burlingame, CA).

Peptide Synthesis. The tyrosine phosphate mimicking amino acid X = *L*-OMT was incorporated into the $EGFR_{988-993}$ segment, D-A-D-E-X-L, using solid-phase synthesis with Fmoc chemistry. The amino acid Fmoc-*L*-OMT$(tBU)_2$-OH was synthesized according to our published method (32). The peptide was prepared using PAL resin (33), DIPCDI/HOBT coupling reagents and 20% piperidine/DMF for Fmoc deprotection. The resin-bound protected peptides were acetylated with 10% 1-acetylimidazole/DMF. The peptide Ac-D-A-D-E-[*L*-OMT]-L-amide (4) was obtained in one step by simultaneous cleavage from the resin and deprotection with TFA containing 5% each (v/v) of ethanedithiol, *m*-cresol, thioanisole and water. The peptides were purified by reverse phase HPLC. Conditions: Vydac C_{18} column (10x250 mm); solvent gradient: A: 0.05% TFA in H_2O, B: 0.05% TFA in 90% acetonitrile in H_2O, gradient (B%): 10-55% over 30 minutes; flow rate: 2.5 mL/minute; UV detector: 220 nm; retention time: 14.5 minutes. FABMS $(M+H)^+$ 868.3 (calcd. 868.3). Amino acid analysis: Asp (1.98), Glu (1.00), Ala (1.01), Leu (1.02); OMT could not be determined by this analysis.



1 pTyr X = O
2 Pmp X = CH_2
3 F_2Pmp X = CF_2

4 *L*-OMT

Figure 1. Structures of pTyr and pTyr mimetics.

Molecular Modelling. Structures of a difluoromethylphosphonate group [$HCF_2PO(O^-)_2$] and a malonate group [$CH_2(CO_2^-)_2$], complexed within the catalytic site of the PTP 1B enzyme (Figure 2A and 2B, respectively) were minimized by an ab initio method using a 3-21G basis set on a CONVEX mainframe computer using GAUSSIAN 92 (34). The geometry of the binding site and mode of binding of the phosphonate were derived from X-ray crystallographic data of a difluorophosphonate-containing inhibitor bound within the PTP 1B catalytic site (35). During the minimization of the difluoromethylphosphonate•enzyme complex, the geometry of the binding site was fixed relative to the X-ray structure, and the geometry parameters and position of the phosphonate were optimized. The minimized geometry of the phosphonate is shown in Figure 2A. In minimizing the complex of the PTP 1B with the malonate structure not only the geometry parameters and position of the malonate were optimized, but also the geometry of the enzyme structure within the binding site during the first 50 hours of CPU time. The minimized malonate complex is shown in Figure 2B.

Cell Line. The Chinese Hamster Ovary (CHO) cell line transfected with an expression plasmid encoding the normal human insulin receptor [CHO/HIRc] used in this study was a generous gift from Dr. Morris F. White, Joslin Diabetes Center, Boston, MA. The cells were maintained in F-12 medium containing 10% fetal bovine serum and were cultured to confluence.

Preparation of Partially Purified Human Insulin Receptors. Membranes from cultured CHO/HIRc cells, overexpressing human insulin receptors were isolated and solubilized with Triton X-100, essentially as described by Liotta et al. (36). Partially purified insulin receptors from solubilized membranes were obtained after passing through a wheat germ agglutin (WGA) column following the method of Brillon et al. (37).

Assay of Insulin Receptor Dephosphorylation by Recombinant PTP 1B. WGA-purified human insulin receptors were autophosphorylated with [γ-^{32}P]ATP as previously described (36), and this ^{32}P-labeled insulin receptor was used as substrate for the assay of PTP 1B activity, essentially following the method described by Burke et al. (21).

Results and Discussion

We (21) and others (16, 17) have shown that peptides bearing phosphonate-based pTyr mimetics can be effective PTP inhibitors. One distinct drawback with such agents in cellular systems is limited bioavailability due to poor transport of the ionized phosphonate moiety through cell membranes. In order to overcome this problem we have drawn from previous work which demonstrated that the malonyl structure can serve as a phosphate mimetic in unrelated enzyme systems (30). A malonyl-derived pTyr mimetic would be advantageous over phosphonate-based analogues in that the malonyl group can be derivatized as its diester form for delivery through cell membranes. Once inside the cell the free diacid form can be liberated by the action of esterases. The compound, *L*-OMT 4 was therefore designed and prepared in a form suitable for incorporation into peptides by solid-phase synthesis (31), and was incorporated in the sequence Ac-D-A-D-E-[*L*-OMT]-L-amide for the present study.

In order to ascertain whether the OMT residue could be accommodated reasonably within the phosphatase catalytic site, we utilized computer assisted molecular modelling to examine how well the malonate structure could be fitted within the catalytic pocket of the PTP 1B enzyme. The previously reported X-ray structure of this enzyme utilized a tungstate ligand, which failed to show the manner in which a phosphate moiety would bind within the active site (38). For modelling purposes we therefore utilized the recently solved X-ray structure of PTP 1B bearing an aryl difluorophosphonate ligand, which approximates the binding of a tyrosine phosphate moiety (35). Based on the geometry of this inhibitor-enzyme complex we determined the binding interaction of a

difluoromethylphosphonate group [(HCF_2-$PO(O^-)_2$] (Figure 2A). The phosphonate is held in place by a cage-like structure of nitrogen atoms. Replacement of the phosphonate structure with the malonate moiety [$CH_2(CO_2^-)_2$] and subsequent minimization, provided the results shown in Figure 2B. Although the malonate structure occupies approximately 12% more volume than the phosphonate, it is easily accommodated within the catalytic site with an enzyme geometry nearly identical to that seen with the phosphonate. These findings suggest that peptides containing the OMT could reasonably bind at the active site, and therefore might be good PTP inhibitors.

Next we examined the effect of the OMT-peptide Ac-D-A-D-E-[*L*-OMT]-L-amide on PTP 1B catalyzed insulin receptor dephosphorylation using ^{32}P-labeled intact insulin receptor as substrate. Figure 3 shows that the OMT-peptide potently inhibited PTP 1B catalyzed dephosphorylation of insulin receptor with a half maximal concentration of 10 μM. This is a significant increase in potency relative to the corresponding peptide which utilized the pTyr mimetic Pmp (IC_{50} = 200 μM (21)). This indicates that OMT is a more effective pTyr mimetic than Pmp in this system.

Currently work is in progress on the preparation of peptides containing OMT protected as its diester. In this non-ionizable form it is anticipated that penetration of OMT peptides across cell membranes will be significantly enhanced, thereby facilitating cellular studies. To the extent that PTPs discriminate with respect to their substrate amino acid sequences, peptide-based inhibitors may offer a degree of selectivity not found with non-peptidic inhibitors. Such selective and cell permeable peptide inhibitors might be useful in understanding the physiological functions of relevant PTPs as well as for affinity purification of these enzymes.

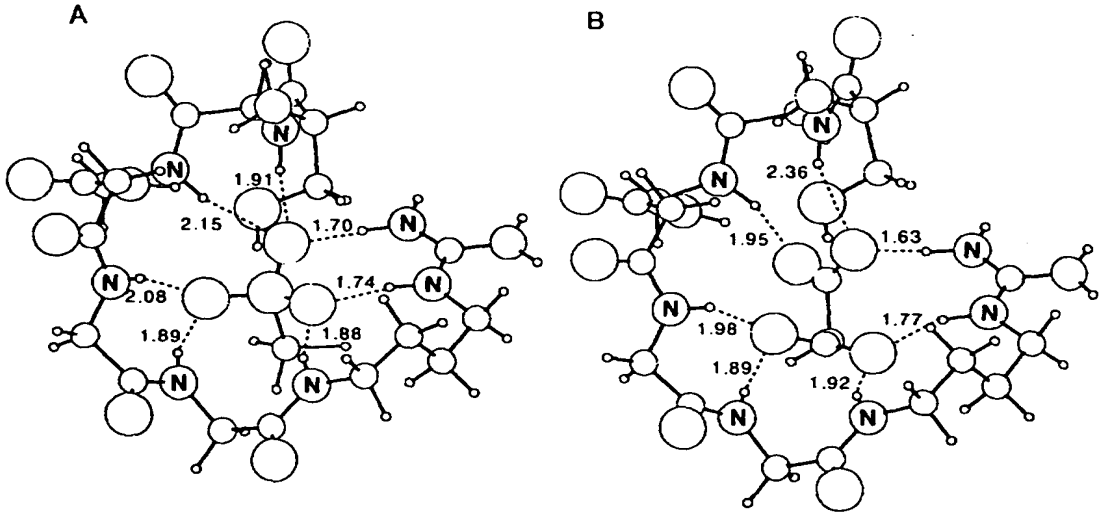


Figure 2. Energy minimized structures of (A) $HCF_2PO(O^-)_2$ and (B) $CH_2(CO_2^-)_2$ bound within the PTP 1B catalytic site. The overall geometry of binding was based on the X-ray structure of an aryl difluorophosphonate inhibitor complexed to PTP 1B (35).

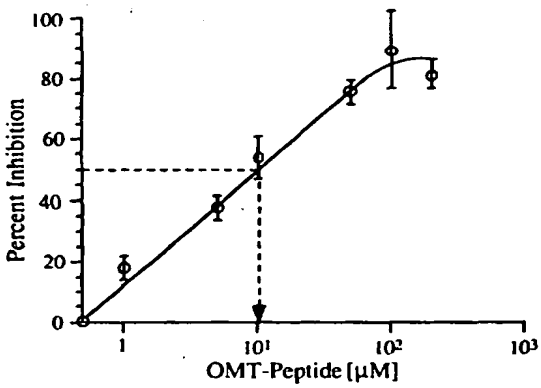


Figure 3. Effect of OMT-peptide on recombinant protein tyrosine phosphatase, PTP 1B activity. WGA-purified human insulin receptors were phosphorylated with [γ-^{32}P]ATP/Mn^{+2} and used as substrate. Dephosphorylation of insulin receptors by recombinant PTP 1B was conducted in the presence or absence of various concentrations of OMT-peptide for one minute at 22 °C as described in "Materials and Methods." Samples were resolved by SDS-polyacrylamide gel electrophoresis under reducing conditions with quantitative analysis of radioactivity on the dried gels by betagen counting with a Betascope 603 Blot Analyzer. Results are the mean ± S.E. of three separate experiments, performed in duplicate.

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